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A COMPARISON OF BIOLOGICAL AND SOME CULTURAL METHODS FOR THE PRIMARY ISOLATION OF *MYCOBACTERIUM TUBERCULOSIS*

By

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INTRODUCTION

Stonebrink (1951) developed a solid culture medium for the primary isolation of *Mycobacterium tuberculosis*, bovine type, incorporating pyruvic acid which was previously shown by Boissevain (1943) to promote the growth of bovine type tubercle bacilli. Although cultural methods of isolation of tubercle bacilli from human sputa have compared well with animal inoculations (Whitehead, 1939; Sasano and Medlar, 1943), the biological test in the guinea pig is still considered superior to any cultural method for the detection of small numbers of tubercle bacilli of the bovine type (Holm and Lester, 1942; Stuart, 1953).

Amongst the mass of literature published on the cultivation of the bovine type tubercle bacillus, there have been very few comparisons of cultural examination with guinea pig inoculation under routine diagnostic conditions. Smith (1953) compared culture on 2 slopes of Dubos penicillin agar with guinea pig inoculation using 312 specimens of bulk milk submitted for biological examination for tubercle bacilli. Thirty of these samples were positive by guinea pig inoculation, and cultural examination recovered tubercle bacilli from 16 (53 per cent). Paterson and Winmill (1950) carried out cultural examination of 46 tuberculous milk samples and recovered tubercle bacilli in Dubos liquid medium from 40 (87 per cent). The 46 samples had been diagnosed as tuberculous by microscopical examination of smears and although very few acid-fast bacilli were seen in some films, these milk samples as a group probably contained comparatively large numbers of bacilli and the chance of recovering organisms on culture would be greater than with a random group of samples. Their results were based on the microscopical examination of stained films prepared from the whole uncentrifuged culture.

We have attempted to evaluate the results obtained in the examination of material for tubercle bacilli under routine diagnostic conditions by the use of Stonebrink and several other culture media with results from guinea pig inoculations.

MATERIALS AND METHODS

Formula No. 126 of Stonebrink (1951) was used. The pyruvate was added as sodium pyruvate instead of the less stable pyruvic acid and the medium prepared as follows.

Salt mixture. Sodium pyruvate, 1·25 g.; potassium dihydrogen phosphate, 0·5 g.; distilled water to 75 ml. Disodium hydrogen phosphate added until pH = 6·5

Egg mixture. (whole egg) 200 ml.

Dye mixture. Crystal violet, 25 mg.; malachite green, 200 mg.; distilled water to 25 ml.

Fresh eggs were washed in soap and water and placed in alcohol for 10 to 15 minutes; on removal the alcohol was allowed to evaporate. The eggs were broken into a sterile bowl and beaten to a smooth consistency with a fork. The salt and dye mixtures were sterilized by steaming for half an hour. All 3 were mixed, filtered through 3 layers of sterile muslin and dispensed in 5 ml. amounts in 6 x $\frac{3}{4}$ inch screw-cap test tubes. The medium was inspissated in a sloped position in the top of a steam sterilizer at 80°C. for half an hour.

The other culture media used were Dorset's egg, Löwenstein-Jensen (Holm and Lester, 1942), Dubos liquid medium (Paterson and Winmill, 1950), Dubos agar and oleic acid-albumin agar (Dubos and Middlebrook, 1947).

The material examined included a variety of samples from different animal species but the work was mainly carried out in connection with the Attested Herds Scheme and the majority of samples were post-mortem specimens from cattle which had reacted to the tuberculin test, together with a number of milk, sputum and uterine mucus samples. These specimens were received, mostly by post, from all parts of England and Wales.

Tissue samples were ground in a mortar with sterile sand and 5 per cent oxalic acid, decanted and the supernatant placed in an incubator at 37°C for half an hour. The fluid was then centrifuged and the deposit suspended in a 0·85 per cent solution of NaCl and again centrifuged. One loopful of the final deposit was used for culturing on each tube and the remainder re-suspended in saline solution for inoculation into 2 guinea pigs. Samples of sputum and uterine discharge were ground in a Griffith's tube with 5 per cent oxalic acid and incubated for half an hour. The centrifuged deposit of milk samples was shaken up in oxalic acid and incubated for the same time. Further treatment was then the same as for other material.

Three weeks after inoculation, single intradermal comparative tuberculin tests were carried out on the guinea pigs, using 0·1 ml. doses of 1 in 25 dilutions of mammalian and avian P.P.D. tuberculins of initial strengths of 2·0 and 0·5 mg. P.P.D. per ml. respectively. Necropsy of the guinea pigs was normally carried out 6 weeks after inoculation.

RESULTS

Trial I

A series of 1623 samples was examined of which 372 (23 per cent) were positive by guinea pig biological tests. One screw-cap tube each of Dorset's egg and Löwenstein-Jensen and 2 tubes of Stonebrink media were inoculated. Tables 1 and 2 show the results obtained after incubation at 37°C for 4 and 6 weeks respectively. The three grades of positive cultures shown in the tables were based on the approximate number of colonies growing in each tube, as follows: + = 1–20, ++ = 21–100, +++ = over 100. The percentages of contaminants shown are percentages of the total number of specimens examined, whilst the percentages of positive cultures are percentages of the number of samples found positive by guinea pig biological tests and finally shown to contain bovine type tubercle bacilli. A culture was considered as contaminated

if non acid-fast organisms had grown in the tube in sufficient quantity to mask the possible growth of tubercle bacilli.

TABLE 1

CULTURAL RESULTS AT 4 WEEKS

Total samples 1623	*Positive by guinea pig inoculation 372*				
Culture medium	*Positive*			*Negative*	*Contaminated*
Dorset's egg	+ 82 ++ 54 +++ 16	152	(41%)	967	504 (31%)
Löwenstein-Jensen	+ 72 ++ 21 +++ 9	102	(27%)	1292	229 (14%)
Stonebrink Tube 1	+ 83 ++ 82 +++ 79	244	(66%)	1216	163 (10%)
Stonebrink Tube 2	+ 90 ++ 80 +++ 44	214	(58%)	1294	115 (7%)

TABLE 2

CULTURAL RESULTS AT 6 WEEKS

Total samples 1623	*Positive by guinea pig inoculation 372*				
Culture medium	*Positive*			*Negative*	*Contaminated*
Dorset's egg	+ 75 ++ 79 +++ 43	197	(53%)	920	506 (31%)
Löwenstein-Jensen	+ 95 ++ 52 +++ 18	165	(44%)	1226	232 (14%)
Stonebrink Tube 1	+ 61 ++ 86 +++ 143	290	(78%)	1170	163 (10%)
Stonebrink Tube 2	+ 77 ++ 86 +++ 105	268	(72%)	1237	118 (7%)

Ninety-six cultures of acid-fast organisms other than tubercle bacilli of the bovine type were recovered from the total of 1623 samples. These consisted of 47 cultures of avian type tubercle bacilli, 2 of human type, one resembling *Mycobacterium johnei* and 46 rapidly growing acid-fast bacteria, 42 of which were isolated from milk or udder from cases of tuberculoid mastitis (Stuart and Harvey,

1951). Since in this trial the recovery of bovine type tubercle bacilli only was being considered, these 96 cultures have been disregarded. They could not reasonably be considered contaminants and therefore have all been included in the total of negative samples except in one case where both an avian and a bovine strain of tubercle bacilli were isolated and the sample has been recorded as a positive.

The higher percentage of positive results obtained on Stonebrink tube 1 as against Stonebrink tube 2 cannot be considered significant as these tubes were not differentiated at the time of sowing, and at the first reading of the cultures there would be a natural tendency to record the tube with the better growth first. There was no further interchange of the 2 tubes of Stonebrink medium at the second reading.

This trial demonstrated the value of Stonebrink medium for the primary cultivation of bovine type tubercle bacilli. Two points in particular were demonstrated, the much higher percentage of positive cultures obtained and the marked reduction in contamination. Reading at 6 weeks, bovine tubercle bacilli were recovered on Stonebrink medium, on an average for the 2 tubes, in 75 per cent of positive cases compared with 53 per cent using Dorset's egg medium. Less than 9 per cent of the Stonebrink slopes were contaminated as against 31 per cent Dorset's egg and 14 per cent Löwenstein-Jensen. The number of +++ cultures obtained on primary isolation in this trial was very much higher on Stonebrink than on the other media, demonstrating a much more rapid and luxurious growth of colonies on the pyruvate medium.

Comparing the results obtained at 4 and 6 weeks, 82 per cent of the positive Stonebrink cultures read at 6 weeks were also positive at 4 weeks, whilst only 77 per cent of the positive cultures on Dorset's egg and 62 per cent on Löwenstein-Jensen at 6 weeks were also positive when read at 4 weeks. This shows that in most cases the appearance of colonies can be expected earlier on Stonebrink than on the other media.

Of the total of 1623 samples examined, 420 were found to contain tubercle bacilli of one of the 3 types. By the combined use of the 2 tubes of Stonebrink medium alone for each sample, tubercle bacilli were recoverd on primary isolation from 345 (82 per cent) of these 420 positive samples. In comparison, the tubes of Dorset's egg and Löwenstein-Jensen media together produced a growth of tubercle bacilli in 282 (67 per cent) of the 420 positive cases.

In no case where bovine type tubercle bacilli were recovered culturally were the biological tests in guinea pigs negative. In 29 positive cases generalised lesions of tuberculosis were found in one only of the 2 guinea pigs inoculated. In some of these cases the second guinea pig was completely negative post-mortem and in others there was enlargement of the lymph nodes local to the site of inoculation, with or without caseation. Most of these samples

were bulk milks or lymph nodes from reactor animals without macroscopic lesions, and probably contained only small numbers of bacilli. Growth was recovered on primary culture in 12 (41 per cent) of these 29 cases.

The 1623 samples received included comprehensive post-mortem specimens from 480 cattle which had been slaughtered as reactors to the tuberculin test, but in which no macroscopic lesions of tuberculosis were found. Laboratory examination showed 50 (10 per cent) to be positive for tubercle bacilli, including 6 avian and 2 human types. Tubercle bacilli were recovered in primary culture from 31 (62 per cent) of these 50 positive samples.

The results of guinea pig tuberculin tests were compared with post-mortem and cultural findings for this series of 1623 samples. It was essential first by special studies to establish what was required of a tuberculin reaction in order to ensure that it was really specific. White guinea pigs must be used as a positive reaction is demonstrated by a ring of erythema with a central purple coloration of the skin. When reactions are large, superficial necrosis of this central area may follow. It was determined that for a definitely positive reaction an area of erythema of 12 mm. diameter or more was required with the avian tuberculin, and 14 mm. or more with the mammalian tuberculin, when read at 24 hours.

In no case where the tuberculin test on a guinea pig was positive for mammalian tuberculosis was the guinea pig found not to be infected post-mortem. In only 8 (2 per cent) of the 374 samples positive for mammalian type did the guinea pigs fail to react to the tuberculin test 3 weeks after inoculation and in 2 of these the guinea pigs were retested at 4 weeks and were found positive. In 22 (47 per cent) of the 47 avian infections, the guinea pigs gave no reaction to tuberculin at 3 weeks but in 9 of these, the guinea pigs were retested at 4 weeks and 7 were found positive. In one case only were the tuberculin tests positive for avian tuberculosis without avian tubercle bacilli being recovered on culture. Rapidly growing acid-fast bacilli were isolated from this sample and the organism was found to have the ability to sensitise guinea pigs to avian tuberculin. One sample containing both avian and bovine strains caused double positive reactions in the 2 guinea pigs at 3 weeks, but the reactions were greater to the mammalian tuberculin.

In the case of samples containing bovine or human type tubercle bacilli, the correlation between the results of guinea pig tuberculin tests and post-mortem findings is very good and a positive report can be given at 3 weeks, when a positive tuberculin test is obtained (Pearson, 1952; Stuart, 1953). Guinea pigs inoculated with material containing avian type bacilli took longer to become sensitised, but nearly 90 per cent gave specific avian reactions by the fourth week after inoculation. These tests help in the typing of avian strains.

Trial II

It had been the custom in this laboratory for several years to use Dubos liquid medium for the primary isolation of tubercle bacilli and after 3 weeks incubation at 37°C to subculture on to Dorset's egg and Löwenstein-Jensen media. As a further trial of a pyruvate medium, 709 samples were examined using this culturing technique except that subcultures were sown after 3 weeks on to single tubes of Dorset's egg, Löwenstein-Jensen and Stonebrink media. Of these 709 samples, 157 (22 per cent) were positive by guinea pig biological tests.

Table 3 shows the results obtained with these 3 solid media after incubation at 37°C for 6 weeks. Fifty-three cultures of other acid-fast organisms were recovered from these 709 samples and consisted of 22 cultures of avian type tubercle bacilli and 31 rapidly growing acid-fast bacteria (30 from milk or udder), all of which have been considered as negative for the purpose of this comparison.

TABLE 3

RESULTS OF SUBCULTURES EX DUBOS LIQUID MEDIUM

Total samples 709	*Positive by guinea pig inoculation 157*				
Culture medium	*Positive*			*Negative*	*Contaminated*
Dorset's egg	+ ++ +++	10 11 48	69 (44%)	445	195 (28%)
Löwenstein-Jensen	+ ++ +++	9 21 21	51 (32%)	522	136 (19%)
Stonebrink	+ ++ +++	41 31 48	120 (76%)	535	54 (8%)

Although this trial did not provide a comparison of the solid media for primary culture, it demonstrated the same two definite advantages of Stonebrink medium for the cultivation of bovine type tubercle bacilli. Firstly, 76 per cent positive cultures were obtained with Stonebrink medium compared with 44 per cent on Dorset's egg medium, and secondly, only 8 per cent of the tubes of Stonebrink medium were contaminated compared with 28 per cent Dorset's egg and 19 per cent Löwenstein-Jensen. The number of +++ cultures on Stonebrink and Dorset's egg media were of the same order, the difference between the results on the 2 media being with those strains which developed relatively few colonies. This indicated that Stonebrink medium is of particular value in cases where organisms are present in small numbers only.

Trial III

Dubos agar and oleic acid-albumin agar solid media were used for subculture from Dubos liquid medium in the examination of another group of 98 samples. Growth on both of these media was better than on Dorset's egg medium, but the results, as shown in table 4, were not as satisfactory as those obtained with Stonebrink medium.

TABLE 4

RESULTS OF SUBCULTURES EX DUBOS LIQUID MEDIUM

Total samples 98	*Positive by guinea pig inoculation 30*				
Culture medium	*Positive*			*Negative*	*Contaminated*
Dorset's egg	+ ++ +++	0 1 12	13 (43%)	55	30 (31%)
Löwenstein-Jensen	+ ++ +++	2 5 3	10 (33%)	67	21 (21%)
Dubos agar	+ ++ +++	2 5 14	21 (70%)	61	16 (16%)
Dubos oleic acid-albumin agar	+ ++ +++	1 5 15	21 (70%)	63	14 (14%)

DISCUSSION

Since these trials Stonebrink medium has been used exclusively for primary culture in the routine examination of material for tubercle bacilli in this laboratory, 5 tubes being sown for every sample. Of 375 specimens so far examined 94 have been found positive, and tubercle bacilli have been recovered on primary isolation from 83 (88 per cent). In those positive cases where tubercle bacilli are not recovered on primary isolation from the original material, further tubes are sown with material from guinea pig lesions and strongly growing cultures are invariably obtained.

Guinea pig inoculation is still used as a routine test, inoculating 1 ml. of suspension into the muscles of the right thigh. The advantage of intramuscular injection over intraperitoneal is that a progression of lesions from the site of inoculation is found post-mortem and also the local abscess which forms does not ulcerate through the skin as is likely with subcutaneous injection. For two reasons a guinea pig is never inoculated with more than one sample. Firstly, avian type tubercle bacilli or acid-fast saprophytes may produce an abscess or enlargement of the lymph nodes local to the site of inoculation in a guinea pig, and if combined with a positive mammalian type

injection on the other side of the same guinea pig, confusion may arise and a false diagnosis be given. Secondly, double inoculations would eliminate the use of guinea pig tuberculin tests as an aid to diagnosis.

The final assessment of any medium for primary culture in the routine examination of samples must be based on the ability to obtain colonies of the organism on a solid medium, as the growth characteristics and morphology of colonies on solid medium are factors usually required for the typing of an organism. A disadvantage of a liquid medium for primary isolation is the time lag of about 3 weeks necessary before subculture on to a solid medium. Also, with Dubos liquid medium the presence of debris from the tissues prevents the use of turbidity as a reliable measure of primary growth and films must be examined microscopically in order to read the cultures. When organisms are seen, it is difficult to be certain that they are actually growing in the liquid medium and it is very doubtful if subcultures on solid media would be readily obtained in all such cases. The results of our trials show that culture direct on Stonebrink medium is as efficient for primary isolation from animal tissues as it is via Dubos liquid medium, and that primary cultivation on Dorset's egg and Löwenstein-Jensen media is rather better than subculture on these media after primary culture in Dubos liquid medium.

Moulds were the main cause of contamination of cultures in these trials and were apparently inhibitory. In some cases a vigorous growth of tubercle bacilli was found in one tube of Stonebrink medium whilst the other had only a single mould colony, but either no growth of tubercle bacilli or only a few small colonies at the other extremity of the slope. The incorporation of crystal violet in an egg medium was found to be satisfactory because of its strong antibacterial action and its anticomplementary colour for yellow which permits an early recognition of colonies of tubercle bacilli.

Results obtained from the primary cultivation of the comparatively small numbers of avian and human types of tubercle bacilli isolated in these trials, and from cultural tests with previously isolated strains, show that both avian and human types grow quite readily on Stonebrink medium. It appears that pyruvic acid, which is a metabolite of glycerol, replaces the latter as a suitable addition to a pigmented egg medium, and the isolation of tubercle bacilli of the avian and human types from animal tissues by the use of Stonebrink medium is at least as efficient as with Löwenstein-Jensen medium.

Stonebrink (1958) has found that where human tuberculous patients have been treated with isoniazid, a pyruvate culture medium, formula No. 211 (Stonebrink, 1952), is considerably more efficient than Löwenstein-Jensen medium for the culture of isoniazid-resistant strains of tubercle bacilli. In a small number of observa-

tions on material from children with primary tuberculosis, the 2 media gave comparable results as regards rapidity of growth and sensitivity. On the other hand, material from adults, generally with tuberculosis of a more advanced type, showed clear differences. Of one series of 93 positive cultures, 53 appeared first on the pyruvate medium, 10 first on Löwenstein-Jensen and 30 on both media at the same time. Growth on the pyruvate medium was not only apparent earlier in most cases, but the colonies also developed to a much larger size than on Löwenstein-Jensen medium. Formula No. 211 contains a slightly increased percentage of the phosphate buffer (KH_2PO_4–Na_2HPO_4), and a reduced quantity of dye, which makes this medium a little more sensitive than No. 126. The medium may not be used for sensitivity tests as the added isoniazid is neutralized by the pyruvic acid.

CONCLUSIONS

The use of Stonebrink and several other culture media has been studied and compared with results of guinea pig inoculations in the examination, under routine diagnostic conditions, of material for *M. tuberculosis*, bovine type.

Stonebrink medium was found to be superior to the other media used on account of the larger number of samples from which tubercle bacilli were recovered, the more rapid and luxurious growth of colonies obtained and the smaller number of contaminated cultures.

Biological tests in guinea pigs were found superior to cultural examination for the isolation of bovine type tubercle bacilli from animal tissues. Tuberculin tests carried out on guinea pigs 3 weeks after inoculation proved reliable and helped in obtaining an early positive diagnosis and also in the differentiation of the avian and mammalian types of tubercle bacilli.

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THE COMPLEMENT FIXATION TEST AND ITS APPLICATION TO THE DIAGNOSIS OF RINDERPEST

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INTRODUCTION

The development of a rapid laboratory test for the diagnosis of rinderpest is of considerable importance. As diagnosis by means of animal inoculation is unsatisfactory in that considerable time is lost waiting for the results, the possibilities of the application of standard serological techniques are worthy of consideration.

Complement-fixing antibodies in the sera of immunised cattle have been demonstrated by Pellegrini and Guarini (1952). Nakamura (1929, 1931, 1936) and Nakamura and Wagatuma (1937) have demonstrated antigens and antibodies in cattle experimentally infected with rinderpest virus, but they have never applied the test as a diagnostic aid.

This paper describes the complement fixation test and its application to the diagnosis of a natural outbreak of rinderpest. At the beginning of July, 1957 a batch of rinderpest-susceptible White Fulani Zebu cattle was purchased by the laboratory to be used for routine vaccine testing. A fortnight after their arrival one animal died showing lesions suggestive of rinderpest and several others were found to be showing typical symptoms. About eight days later a disease resembling rinderpest appeared in the laboratory herd of sheep. In view of the unusual nature of this occurrence the outbreak was extensively studied and has been described in detail by Johnson (1957). Prescapular lymph glands were collected from the cattle and sheep for the demonstration of antigen and serum samples were collected for the demonstration of antibodies.

MATERIALS AND METHODS

Diluent. Throughout the tests 0·9 per cent saline buffered with M/150 phosphate at pH 7.2 was used as diluent.

Complement. Normal guinea pig serum, preserved by freeze-drying, was the source of complement, the same batch of freeze-dried complement being used in all the tests.

Haemolytic system. A 2·2 per cent suspension of washed goat red cells sensitised by 4 MHD of haemolysin was used.

Antigen. A known positive antigen (PA) and a corresponding normal antigen (NA) were prepared from dried samples of prescapular lymph glands from infected and normal Japanese calves. These antigens were extracted from the dried tissues in a mixture of $\frac{N}{50}$ sodium hydroxide and normal saline and adjusted to pH 7.2 to 7.6. The antigens were used

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in a dilution of 1:200. The method of preparing the dried antigen and its extracts is described by Nakamura (1957). In all the tests for the demonstration of local antigen, fresh prescapular lymph gland was used. A small piece of gland was ground up with saline to make a ten per cent suspension. This was boiled in a water bath for 30 minutes and centrifuged for 15 minutes at 4,000 r.p.m. The supernate constituted a 1:10 dilution of heat stable antigen.

Antibody. Lyophilised sera were prepared in Japan, unheated hyperimmune bovine serum (PS) being used as antibody in parallel with unheated normal bovine serum (NS). Both had been previously balanced with regard to their anticomplementary activity. All the local sera tested were unheated and diluted to 1:10.

Demonstration of antigen by the antigen dilution method. The method used was that described by Nakamura (1936, 1957) which was, briefly, as follows. The antigen (0·2 ml.) was diluted in a twofold series in nine tubes plus three control tubes for normal serum. The hyperimmune (PS) and normal (NS) sera in dilutions of 1:20 were added in 0·1 ml. amounts. Then 0·1 ml. of complement at a dilution of 1:12 was added to each tube. This represented 1·3 haemolytic doses in the presence of NS and 1.6 haemolytic doses in the presence of PS, i.e., an excess of complement of 33 per cent and 66 per cent respectively. The tubes were placed overnight in a refrigerator (4°C). After this fixation period, 0·1 ml. of the sensitised red cell suspension was added to each tube and the tubes placed in a water bath (37°C) for 30 minutes. A tentative reading was obtained immediately after the tubes were removed from the bath. The tubes were placed again in the refrigerator overnight and the final results were read on the following day.

Demonstration of antigen by the complement dilution method. This method was originally applied to the demonstration of weak antigen in the tissue of embryos infected with avianised rinderpest viruses (Nakamura and Miyamoto, 1953) and later to lymph nodes of rabbits infected with lapinised virus (Nakamura, 1957).

The principle of the test is the measurement of the complement-fixing capacity of a definite amount of antigen and antibody. Antibody and antigen are used, therefore, in fixed amounts and complement is diluted in series. The test consists of two parallel series of ten tubes containing normal serum (NS) and hyperimmune serum (PS) respectively. Equal parts of serum in a dilution of 1:20 and antigen in the required dilution were mixed and 0·1 ml. of the mixture was added to each tube. Complement was diluted in a 4/5 series and distributed in 0·1 ml. amounts. After a fixation period during which the tubes were placed overnight in a refrigerator (4°C), 0.05 ml. of sensitised red cells was added and the tubes were placed in a water bath at 37° for thirty minutes. They were again refrigerated overnight and the degree of haemolysis was then read and expressed as a percentage by means of visual comparison with a standard haemolysis series prepared simultaneously from the same blood cells as were used in the test.

Demonstration of antibody by the complement dilution method. The method of demonstrating antibody involved the same principle as that of the complement dilution method which was applied to the demonstration of antigen described above. This test has been applied mainly to bovine

sera (Nakamura, Kishi, Kiuchi and Reisinger, 1955) and to rabbit sera (Nakamura, Kishi and Kiuchi, 1953).

RESULTS

Antigen Dilution Method

In using this method for the demonstration of antigen, ten bovine prescapular lymph glands were tested. Five of these (LG/B1 to LG/B5) gave strongly positive results. The remaining five samples (LG/B6 to LG/B10) were completely negative. The normal Japanese antigen exhibited complete haemolysis throughout while the positive Japanese antigen gave similar results to that of LG/B1, i.e., complete inhibition of haemolysis in the first eight tubes.

Thirty-four sheep were tested by the same method; 26 gave completely negative results, six were positive (LG/S1, LG/S2, LG/S3, LG/S4, LG/S5 and LG/S8) and two gave doubtful reactions (LG/S6 and LG/S7.) All positive reactions were much weaker than those observed in cattle and in no instance did complete inhibition of haemolysis occur. In order, therefore, to determine the strength of these positive reactions more accurately, the more sensitive complement dilution method was used. Before applying this method, however, it was necessary to test the sheep antigen by the antigen dilution method in order to determine the solution at which each antigen would give optimal results. It is known that a strict optimal relationship exists between definite amounts of bovine antigen and antibody (Nakamura, 1929) and it was expected that this phenomenon would apply to the reaction of sheep antigens. The results obtained by the antigen dilution method confirmed this.

Specific reaction was estimated by a comparison of the results in the NS and PS series in a limited series of four to six consecutive tubes. It was found by experience that the most satisfactory results were obtained by taking an 80 per cent end-point. The limited series was therefore chosen to give an average percentage haemolysis in the NS series of 80 per cent. The fixation titre (FT) is the ratio of the point at which 80 per cent of blood cells remain undestroyed as a consequence of the fixation of complement compared with the total amount of blood cells which were haemolysed in the NS series. This ratio, expressed as a percentage, is obtained from the following formula: $FT = \frac{Hn - Hp}{Hn} \times 100$, where Hn = percentage haemolysis in the normal series and Hp = percentage haemolysis in the positive (hyperimmune) series.

The interpretation of the reaction is as follows: 0 to 10 per cent, negative; 11 to 20 per cent, doubtful; 21 to 100 per cent, positive. Eleven antigen samples were tested by this method and consisted of the six positive samples, the two doubtful samples and three samples which had given negative results to the antigen dilution method. The six positive antigens again gave positive results, but the individual differences were much more apparent. Thus, LG/S2 was the

TABLE 1

EXAMPLES OF ANTIGEN DEMONSTRATED BY THE COMPLEMENT DILUTION METHOD

Antigen	*Serum*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*FT%*
Blank	Blank	100	100	100	100	100	100	95	50	20	5	
Blank	NS	100	100	100	95	70	20	10	0	0	0	—19
	PS	100	100	100	100	95	80	70	40	20	5	
LG/S2 1 : 20 .. .	NS	100	100	100	100	95	80	50	20	5	0	100
	PS	20	5	0	0	0	0	0	0	0	0	
LG/S5 1 : 20 .. .	NS	100	100	100	100	95	80	60	30	10	0	76
	PS	95	60	50	30	20	10	0	0	0	0	
LG/S8 1 : 10	NS	100	100	100	100	90	60	30	5	0	0	24
	PS	100	100	95	70	60	30	10	0	0	0	
LG/S9 1 : 10	NS	100	100	100	100	95	80	80	50	30	10	—2
	PS	100	100	100	100	95	90	80	50	40	20	

LG/S = Sheep lymph gland NS = Normal bovine serum PS = Hyperimmune bovine serum FT = Fixation titre

TABLE 2

EXAMPLES OF ANTIBODY DEMONSTRATED BY THE COMPLEMENT DILUTION METHOD

Serum	*Antigen*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*FT%*	FT% *corrected*
Blank ..	Blank	100	100	100	100	100	95	80	50	30	20		
Blank ..	NA	100	100	100	100	100	95	90	80	70	30	19	0
	PA	100	100	100	100	100	95	70	60	30	20		
S/S12 ..	NA	100	100	100	95	90	80	50	40	20	—	100	81
	PA	95	50	10	0	0	0	0	0	0	—		
S/S13 ..	NA	100	100	100	100	100	95	90	40	20	—	43	24
	PA	100	100	100	100	95	70	20	0	0	—		
S/S14 ..	NA	100	100	100	100	100	95	90	60	30	—	64	45
	PA	100	100	100	80	70	20	0	0	0	—		
S/S15 ..	NA	100	100	100	95	95	90	80	30	—	—	15	—4
	PA	100	100	100	95	90	70	40	20	—	—		

S/S = Sheep Serum NA = Normal antigen PA = Positive antigen FT = Fixation titre

strongest (FT 100 per cent), followed in descending order by LG/S1, LG/S5, LG/S4, LG/S3 and LG/S8 (FT: 82, 76, 63, 38, to 24 per cent respectively). The two doubtful samples LG/S6 (FT–3 percent) and LG/S7 (FT–3 per cent), were negative. The three negative samples again gave negative results. The controls were normal bovine antigen (NA) which gave an FT of 0 per cent, and positive local antigen prepared from bovine sample LG/B3. The latter was the weakest of the five positive bovine antigens as judged by the antigen dilution method and gave an FT of 100 per cent.

Complement Dilution Method

Twenty-two sheep and four bovine serum samples were tested for the presence of antibody. The results (Table 2) were calculated by the method already described with a slight modification to compensate for an unbalanced pair of positive (PA) and negative (NA) antigens. In a well balanced test pair of sera or antigens the normal should be equally or preferably a little more anticomplementary when compared with the positive. Where NS is obviously more anticomplementary than PS the FT is negative (Table 2). In the case of the NA and PA pair, however, the reagents were unfortunately not well balanced, so that when tested in the absence of sera they gave an FT of 19 per cent. To compensate for this in the calculation of the FT of the samples it was decided to deduct this figure from the result calculated by the usual method. In Table 2 both the actual FT and the corrected FT figures are given.

Of the twenty-two sheep sera tested, eleven were positive and eleven were negative. Four samples gave a strong reaction, S/S12, S/S17, S/S3, S/S20 giving an FT of 81, 75, 75, 73 per cent, respectively, and the remainder, S/S13, S/S16, S/S18, S/S19, S/S14, S/S21, S/S22, gave weak positive reactions, i.e., an FT of 24, 39, 39, 35, 45, 21 and 43 per cent respectively. Ten of the eleven sheep giving positive antibody titres were negative to the test for the demonstration of antigen, the exception being S/S3 which gave a weak antigen response (FT 38 per cent) and a strong antibody titre (FT 75 per cent). Apart from S/S3, those sheep in which antigen was demonstrated were negative when tested for antibody.

Only four bovine samples were available for antibody testing. Three came from cattle which gave strong antigen titres and one sample was from the group which was negative to the antigen demonstration. The results of the test on the four sera were completely negative.

DISCUSSION

The lymph glands of the five cattle in which antigen was demonstrated all gave strong positive reactions. In addition, there appeared to be a correlation between the stage of infection, as decided by the character of the lesions, and the results obtained from the test for the demonstration of antigen. Thus, the five animals in which the

reaction was positive were in the active stages of infection, while, of the five animals in which the results were negative, four were in the later stages. The remaining animal failed to show any characteristic lesions and hence was either not infected or was in the incubative stage of the disease. These results are in accordance with those observed by Nakamura and Wagatuma (1937) who analysed the relationship between the course of the disease and the results of the CF test in 23 fatal cases of rinderpest in Korean calves, which were inoculated simultaneously with virus and immune serum. They found that four cases which died within six days of the onset of fever were all positive to the CF test. Of 13 cases which died between the sixth and ninth day, eight (61 per cent) were positive and five (39 per cent) were negative. All the remaining six cases which died between the tenth and twelfth day were negative.

In sheep, however, the picture was somewhat different. All six positive sheep lymph glands gave a much weaker reaction than did the cattle lymph glands. This suggests that the intensity of infection, or the propagation of the virus, was much lower in sheep than in cattle. It is the generally accepted view (Beaton, 1954; Curasson, 1942; Plowright, 1952; Scott, 1955) that sheep are much less susceptible to rinderpest than cattle. In this outbreak, however, the impression received was that the disease in sheep was much more severe than is generally recognised. There were many cases with severe lesions and the mortality was high. The factors responsible for this were obscure.

The relationship between the lesions and the demonstrable CF antigen was less evident in sheep than in cattle. This gave the impression that in sheep the infection proceeded more irregularly and so the lesions were less indicative of the course of infection. Furthermore, as mentioned above, there was a suspicion that the disease was modified by some factors other than the pure effect of the virus. This has been discussed by Johnson (1957). One further point to be noted is that in many sheep the lesions were suggestive of the acute stage of infection, but no antigen was demonstrated. Since, however, even the strongest reaction obtained was only a little above the lowest demonstrable level, it may be presumed that in some of the cases at least the antigen had failed to reach this demonstrable level. In the present outbreak, eleven sheep gave positive antibody titres. Four of these showed no lesions and were considered to have had an inapparent or slight infection. Six showed lesions suggestive of the later stages of infection. The remaining one showed lesions resembling the earlier stages of the disease.

The relationship between the antigen and antibody response in the animals tested may be summed up as follows. Five cattle and three sheep were antigen-positive and antibody-negative, ten sheep were antigen-negative and antibody-positive, one bovine and nine sheep completely negative and one sheep showed a positive antigen and

antibody response. The first two groups are self-explanatory. In the third group various interpretations may be applicable, e.g., they may have been in the incubative stage, or the titres of antigen or antibody may not have reached a demonstrable level at the time of testing. In the last group, one sheep (S3) had a low antigen titre (FT 38 per cent) and a relatively high antibody titre (FT 75 per cent). This finding apparently suggests the occurrence of a period in which both antigen and antibody coexist in an animal.

Throughout the tests the reagents used for the demonstration of antigen and antibody originated from a Japanese strain of virus. This strain, designated "O", is a classic strain originally isolated in Japan and passaged in Japanese and Korean calves for over 40 years. Nakamura (1931) isolated another strain, "K", in Korea and as a result of detailed cross tests, using hyperimmune sera against both strains, the "K" strain was differentiated from the "O" strain thus proving the existence of serological plurality in the rinderpest virus. It would be of interest to know whether the wild strain isolated here is in any way related to the Japanese and Korean strains. It was also interesting to find that the antigens prepared from the lymph glands of Zebu cattle infected in the field gave the same degree of serological reaction to the Japanese serum as did the antigens prepared from the lymph glands of Japanese calves which were infected experimentally.

CONCLUSIONS

As a result of using material from a natural outbreak of rinderpest, it is suggested that the complement fixation test for the demonstration of antigen and antibody is a satisfactory method for the diagnosis of the disease. Although the test is not always effective in confirming infection in the individual animal it does enable a diagnosis to be made rapidly on a herd basis. It can also be applied effectively to animals which have some degree of resistance, e.g. Zebu cattle and sheep.

It must be borne in mind that if the test is to be successful the stage of infection at which the samples are obtained is important and fresh lymph glands are necessary for the preparation of the antigen. These two factors must be taken into account before applying the test to the diagnosis of a natural outbreak.

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METABOLIC CHANGES IN CATTLE NEAR THE TIME OF PARTURITION

1. Hepatic Fat and Alkaline Phosphatase activity of Liver Homogenates

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INTRODUCTION

The appearance of stainable fat in the hepatic cells of cattle and an increase in the alkaline phosphatase activity of these cells was briefly described in a preliminary communication (Ford, 1957).

The present paper describes more fully the investigations on which the above communication was based. The observations made were the serum bilirubin and serum alkaline phosphatase levels, an examination of fixed liver biopsy material stained for general morphology, fat and alkaline phosphatase and the determination of the dry weight, protein content and alkaline phosphatase activity of fresh liver biopsy material.

MATERIALS AND METHODS

Animals and sampling. Shorthorn cattle from the milking herd attached to this Institute were used. The animals were managed and fed according to commercial farming standards. In the first series of observations liver biopsy was carried out by a modification of the method of Loosmore and Allcroft (1951) at approximately three-week intervals commencing two to three months before the expected date of parturition and continuing for a similar period after parturition. The sample of liver was placed on filter paper to allow drainage of blood, a portion was placed in neutral formol-saline and the remainder was weighed with the minimum of delay. At the same time as the biopsy a sample of blood was withdrawn from the jugular vein, allowed to clot and the serum was separated. In the later stages of the investigation the frequency of biopsy was increased to once every ten days and when parturition was imminent the interval between biopsies was reduced to seven days.

Analytical methods. Serum alkaline phosphatase was determined by the method of King and Armstrong (1934) and serum bilirubin according to Dangerfield and Finlayson (1953). Biopsy material after fixation in neutral formol-saline was stained with haematoxylin and eosin for general morphology, with Sudan III for neutral fat and by a modification of the method of Gomori (1939) and the coupling azo-dye method described by Pearse (1954) for alkaline phosphatase. A weighed portion of the fresh liver was immediately homogenised in 0.9 per cent sodium chloride solution for estimation of the alkaline phosphatase activity. The dry matter content of the remainder of the liver was determined after drying overnight in an oven at 100°C. The dried material was then digested with concentrated sulphuric acid and copper sulphate and the nitrogen

determined by the micro-Kjeldahl method, the ammonia being distilled over in a Markham apparatus. Protein was calculated as N x 6·25.

RESULTS

Serum

Alkaline phosphatase and bilirubin levels showed no changes associated with parturition. Whilst the serum levels of individual animals remained fairly constant over the period of the observations there were variations in levels between animals especially in the case of alkaline phosphatase (Table 1). The serum alkaline phosphatase levels fall within the range reported by Allcroft and Folley (1941), and the serum bilirubin levels were comparable to those obtained by Garner (1953) using the method of Malloy and Evelyn (1937).

TABLE 1

SERUM ALKALINE PHOSPHATASE AND BILIRUBIN LEVELS

Cow	*1*	*2*	*3*	*4*	*5*
Alkaline Phos. K.A. units /100 ml.	10·2 ± 2·4 (7)	18·9 ± 9 (7)	22 ± 4·5 (9)	5·2 ± 1·5 (5)	4·3 ± 1·1 (6)
Bilirubin mg./100 ml.	0·26 ± ·04 (6)	0·38 ± ·07 (6)	0·33 ± ·07 (8)	0·24 ± ·13 (4)	0·33 ± ·09 (6)

Liver

The only detectable change in the general morphology of the liver sections during the period of examination was the appearance of stainable fat in the hepatic cells of a number of biopsies. Discrete globules of fat in the Kupffer cells were also present in the majority of biopsies. Table 2 shows the occurrence of hepatic cellular fat in relation to the time of year and Table 3 shows the relationship that the same results bear to parturition. It is clear that the histologically detectable fat appears near the time of parturition and in most of the animals the change occurred in the early stages of lactation.

In the case of the first cattle sampled the frequency of the biopsies provided only one or sometimes two fat-containing samples, but when biopsies were obtained at shorter intervals the duration of the fatty change was more clearly demonstrated and varied from two to six weeks in four cattle. Figs. 1 to 3 and 4 to 6 depict fat-stained liver sections from cows four and five where the biopsy interval was approximately three weeks and Figs. 7 to 12 show a series of liver sections from cow ten obtained at shorter intervals.

It was not possible to detect histologically, by either of the methods used, sites of alkaline phosphatase activity in the hepatic cells despite many modifications in technique and full use of positive

TABLE 2

STAINABLE HEPATIC CELLULAR FAT (SUDAN III)

Cow	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	April	May	June	July	Aug.
1 ..		— —	-	+ + +	-						
2 ..		- —	+ +	—	— -	—					
3 ..				-- -	—	—	+				
4 ..				— —	— —	—	-	+ — +			
5 ..				—	—	—	+ +	- -			
6 ..								—	— —	+	—
7 ..					— —	.	- — + +	— tr	—		
8 ..					- —	— — +	+ + —	— —	.	—	
9 ..					—	- —	— — —	— tr +	—	+	
10 ..	— — — —	+ + + + + +	+ + - +								

— = no stainable fat in hepatic cells

tr = few fine droplets in small number of hepatic cells

\+ = well marked globules in some hepatic cells

+ + = well marked globules throughout the lobules

TABLE 3

STAINABLE HEPATIC CELLULAR FAT

	Weeks before parturition														*Weeks after parturition*												
Cow	*14*	*13*	*12*	*11*	*10*	*9*	*8*	*7*	*6*	*5*	*4*	*3*	*2*	*1*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*
1										—			—		—				+				+				—	
2								—			—			+				—			—			—			—	
3			—			—			—			—				+												
4	—	—			—			—			—				—			+			—							
5							—			—				—		+					—			—				
6								—			—		—		+			—				—						
7					—		—			—		—	—	—	+	+	—			tr		—						—
8								—		—	—	—		+	+	+	—		—		—		—		—		—	
9					—		—		—	—			—	—	—		—	tr		+		—			+	—		
10											—	—		—	+	+	+		+	+	—							

— = no stainable fat in hepatic cells

tr = few fine droplets in small number of hepatic cells

\+ = well marked globules in some hepatic cells

\+ + = well marked globules throughout the lobules

TABLE 4

PROTEIN CONTENT OF LIVERS EXPRESSED AS % OF WET WEIGHT

Group	*1*	*2*	*3*	*4*
Days from parturition ..	40 – 21	20 – 1	1 – 20	21 – 40
Number of observations ..	3	5	8	7
Mean value	14·5	15·8	16·5	18·1
Standard deviation ..	±1·5	±·7	±2·2	±2·0

Degrees of freedom ..	6	11	13
't'	1·8	0·65	1·35
P	<·1	<·6	<·2

The comparisons are of the two consecutive means above each comparison column. Comparing Groups 1 and 4, D.F. = 8, 't' = 2·43 and P = <·05

TABLE 5

ALKALINE PHOSPHATASE ACTIVITY OF LIVER HOMOGENATE

K.A. Units/100 g. liver

Group	*1*	*2*	*3*	*4*	*5*	*6*
Days from parturition ..	60 – 41	40 – 21	20 – 1	1 – 20	21 – 40	41 – 60
Number of observations	9	13	16	23	15	7
Mean value	366	376	433	558	388	392
Standard deviation ..	±190	±149	±165	±156	±245	±83

Analysis of differences between means

Groups compared ..	1 & 4	2 & 4	3 & 4	4 & 5	4 & 6	
Degrees of freedom ..	30	34	37	36	28	
't' ..	2·95	3·41	2·40	2·61	2·68	
Probability ..	<·01	<·01	<·05	<·02	<·02	

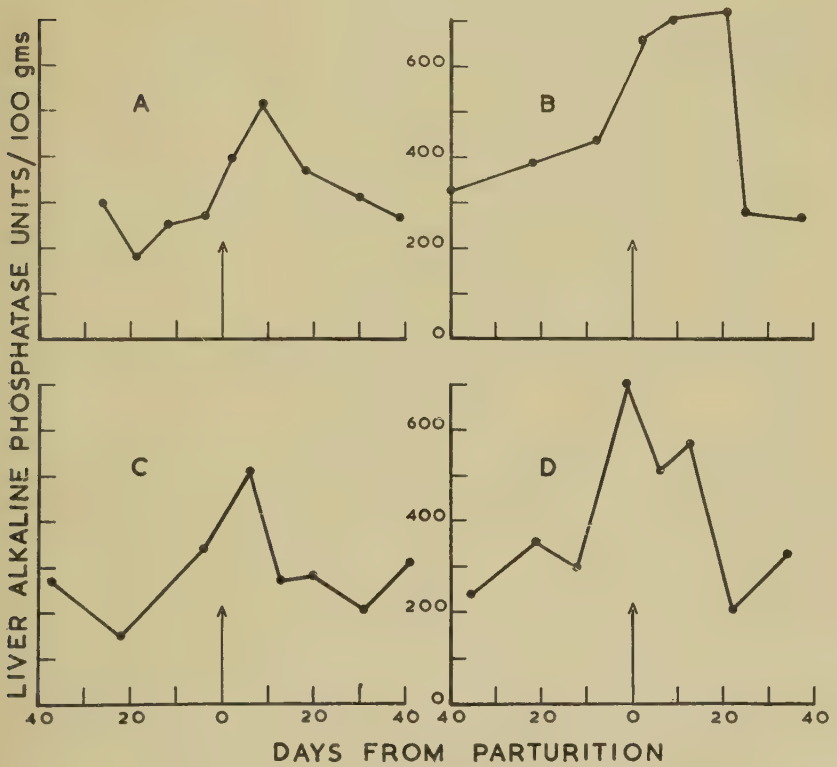
control material. It would appear that the cellular enzyme concentrations were below the threshold for the methods employed.

The dry weight of the liver did not change significantly during the period of the observations, the mean value being 27·7 ± 2 per cent for 24 samples. Table 4 suggests there may be a rise in the mean protein content of the liver during the period of sampling, but the difference between the group of samples taken 40 to 21 days before

parturition and the group taken 21 to 40 days after parturition was significant only at the 5 per cent level.

Table 5 giving levels of alkaline phosphatase activity of liver homogenates in relation to the time of parturition, shows that activity in the immediate post-parturient period was significantly higher than the levels in late pregnancy and that activity falls again as lactation proceeds. Alkaline phosphatase changes in the livers of four individual animals are shown in Fig. 13.

Fig. 13



Examples of hepatic alkaline phosphatase changes in individual cattle

DISCUSSION

This investigation has shown well-marked changes in the liver of cattle close to the time of parturition. One of these is the appearance of stainable fat in the hepatic cell and the disappearance of this fat as lactation proceeds. Gaiger and Davies (1938) stated that there is a physiological fatty infiltration of the liver during pregnancy and lactation and Wolterstorff (1921), who had examined liver from slaughtered pregnant cattle, found that whilst fat commonly occurred in the endothelial cells of the sinusoids, fine droplets appeared in the hepatic cells in the latter third of pregnancy

and by the last month the majority of hepatic cells contained fat droplets. The comments of Gaiger and Davies (1938) and the results reported by Wolterstorff (1921) suggest that fat was found in hepatic cells over a longer period than in the present investigation in which two positive biopsies were obtained in the last week of pregnancy and thirteen of the remaining seventeen occurred in the first five weeks of lactation.

Shaw and Leffel (1949) reported higher fat content in the livers of cattle after parturition compared with late pregnancy in animals that had suffered a sharp reduction in diet. They attribute the increase in liver fat to the drop in food intake, disregarding the possible effects of the change from pregnancy to lactation. In the present series of animals, as in all normally managed dairy herds, increased rations were offered from late pregnancy onwards to encourage full milk production.

As far as the writer is aware the literature contains no information on changes in enzyme levels of bovine liver during pregnancy or lactation. Folley and Greenbaum (1947) showed that the arginase activity of rat liver rises in late pregnancy and remains at a high level during lactation, and suggested that as the enzyme is concerned with the deamination of amino-acids an increase in activity reflects an augmentation of hepatic gluconeogenesis. The relative non-specificity of alkaline phosphatase activity makes it difficult to interpret the significance of the increase in enzyme activity in early lactation. It is possible that there is an increase in phosphorylation associated with increased carbohydrate metabolism, a reflection of the latter being the parturient hyperglycaemia in cattle described by Godden and Allcroft (1932) and Van Soest and Blosser (1954), and also the depletion of liver glycogen at parturition recorded by Shaw and Leffel (1949). De Angelis, Vignolo and Barsantini (1955) studied the endocrine regulation of liver arginase activity in the cat. They showed that activity of the enzyme was increased by the adrenocorticotrophic hormone of the pituitary gland and also by the hormones of the adrenal cortex, in particular by cortisone.

The regulation of the mobilization of depot fat to the liver has been reviewed by Levin and Farber (1952) who state that increased secretion of growth hormone by the anterior pituitary is responsible, but that adrenal cortical steroids are also necessary. If alkaline phosphatase activity is subject to the same hormonal regulations as is arginase activity, the above evidence could suggest that the liver changes in parturient cattle are stimulated by increased pituitary activity coupled with a response from the adrenal cortex. Indirect evidence of such increased activity in cattle at the time of parturition has been provided by Kerr, Robertson and McGirr (1951) and, in addition, Paterson (1957) has shown that cattle have raised plasma 17-hydroxycorticosteroids immediately before parturition.

This interesting hypothesis is being studied experimentally by investigating the effects of the administration of pituitary and adrenal hormones on liver metabolism in non-pregnant cattle.

CONCLUSIONS

There are no significant changes in serum bilirubin, serum alkaline phosphatase or in the dry matter of the liver of cattle at parturition.

The protein content of the liver in the period 21 to 40 days after calving is slightly higher than in the 40 to 21 days before calving. The alkaline phosphatase activity of liver homogenate is significantly higher in the first 20 days after parturition than in the later stages of pregnancy and there is a significant fall as lactation proceeds.

Stainable fat appears in hepatic cells in the first five weeks of lactation and is removed as lactation proceeds.

The mechanism of these changes is discussed and increased pituitary-adrenal activity at parturition is suggested as a possible cause.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Liver section from cow 4, one week after parturition. Stainable fat absent

Fig. 2. Liver section from cow 4, four weeks after parturition. Large globules of fat in hepatic cells

Fig. 3. Liver section from cow 4, seven weeks after parturition. Stainable fat absent

Fig. 4. Liver section from cow 5, one week before parturition. Stainable fat absent

Fig. 5. Liver section from cow 5, two weeks after parturition. Large globules of fat in hepatic cells

Fig. 6. Liver section from cow 5, seven weeks after parturition. Stainable fat absent

Fig. 7. Liver section from cow 10, one week before parturition. Stainable fat absent

Fig. 8. Liver section from cow 10, one week after parturition. Numerous globules of fat in hepatic cells

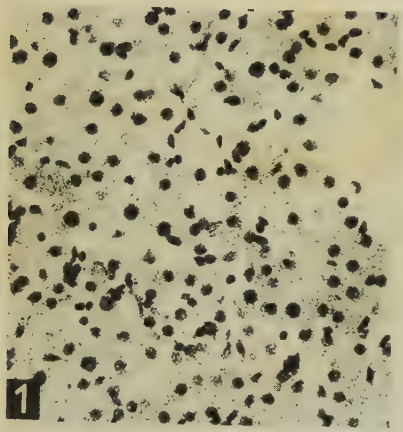
Fig. 9. Liver section from cow 10, three weeks after parturition. Numerous large globules of fat in hepatic cells

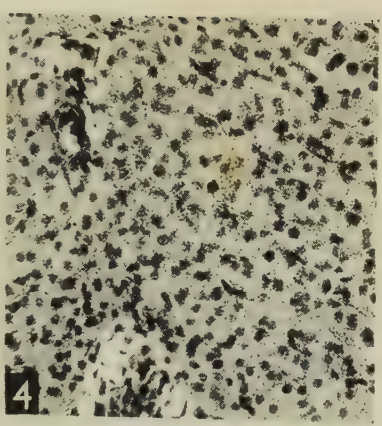
Fig. 10. Liver section from cow 10, five weeks after parturition. A small number of fat globules in hepatic cells

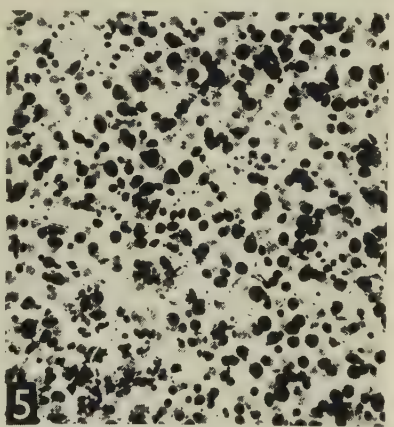
Fig. 11. Liver section from cow 10, six weeks after parturition. Very few large fat globules in centre of section

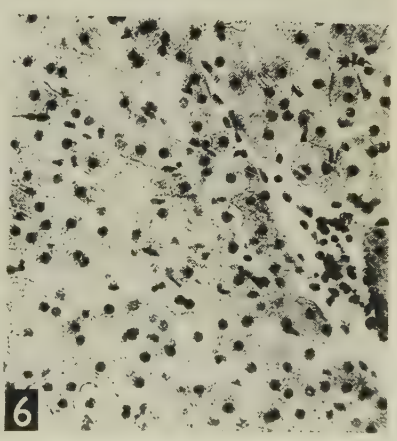
Fig. 12. Liver section from cow 10, seven weeks after parturition. Stainable fat absent

All sections frozen, stained Sudan III and haemalum, × 160









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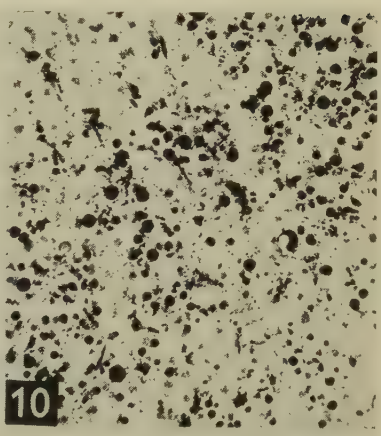
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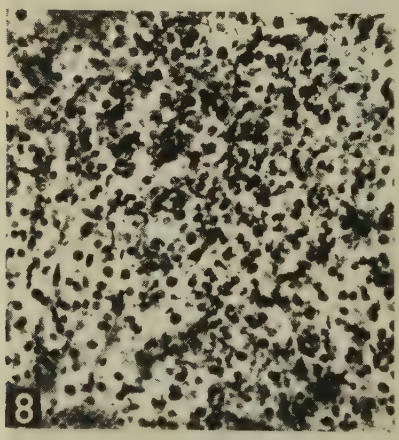
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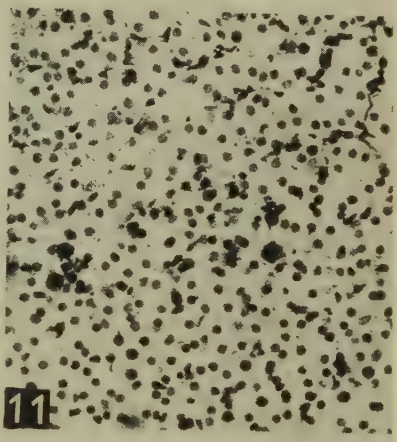
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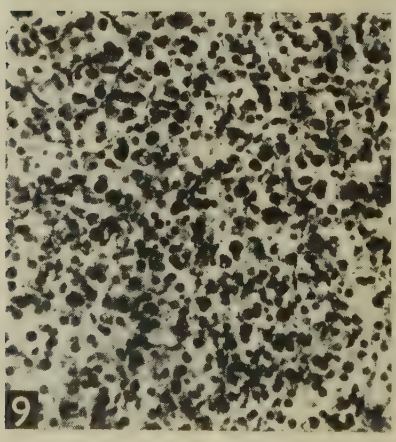
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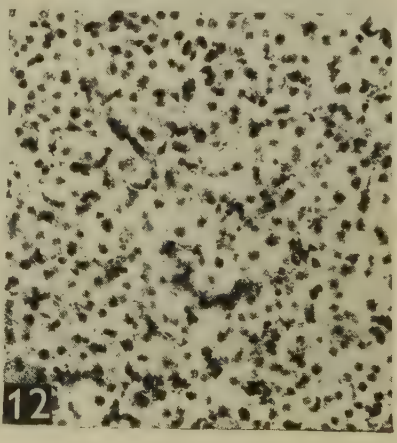
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THE BLOOD GROUPS OF THE PIG

VI. Red Cell Antigens other than the A-O System

By

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INTRODUCTION

Previous work from these laboratories on the production of iso-antibodies in pigs (Goodwin, Saison and Coombs, 1955; Goodwin and Saison, 1956) and on haemolytic disease of the new-born (Goodwin, Hayward, Heard and Roberts, 1955, 1956; Goodwin and Saison, 1957; Goodwin, 1957) was carried out without knowledge of the individual antigen-antibody systems involved. Now, however, the more long-term study of pig blood groups has reached a stage where some of these systems have been characterized and progress can be made in relating them to the disease in the field.

From the sera of six sows that had had litters affected with haemolytic disease, the iso-antibodies have been separated to give testing reagents of known specificity and, by this means, eleven blood-group antigens have so far been defined. With eight of these antigen-antibody systems the optimal serological test has also been determined. As the testing sera became available only progressively, they have not often been fully applied to field cases of the disease. Nevertheless, from the limited blood-group patterns of the sows and boars, aided sometimes by an analysis of the sow's serum, it has been possible to enumerate the antibodies that could have been involved in some of the affected litters.

The frequencies of these blood-group antigens in different breeds of pigs have also been studied; but these findings, together with any light they may shed on the observation of Goodwin and Saison (1956) that there is a breed difference in iso-antibody response after vaccination with crystal violet vaccine, will be the subject of a later paper.

MATERIALS AND METHODS

Sera. The sera analysed contained iso-antibodies at high titre and they were obtained from sows that had been vaccinated with crystal violet swine fever vaccine and then had piglets affected with haemolytic disease. In the tests, the sera were used unheated. No haemolysis was observed when using these undiluted sera on pig cells.

Red blood cells. Pig blood was obtained from two sources; a constant panel of farm pigs and the slaughterhouse. In both cases it was taken into either an acid-citrate-dextrose or a tri-sodium citrate medium. Slaughterhouse blood could be obtained in large volumes and, after being fully grouped with all available serum reagents, it was used for absorptions; it had the disadvantage, however, of being available for only about 1 week. Before testing, the red cells were washed and suspended in 0·85

per cent saline, maintained at pH 7·2 with phosphate buffer (final molarity 1/1500).

Agglutination and anti-globulin sensitization tests. The direct agglutination test, indirect anti-globulin sensitization test (I.S.T.) and the agglutination test using cells treated with trypsin (Morton and Pickles, 1951) were performed as before (Saison, Goodwin and Coombs, 1955), except that all incubations were at 37°C. In some of the direct agglutination tests, noted in the text, the incubated serum-cell mixtures were centrifuged at 3000 r.p.m. for 30 sec. before being examined microscopically for agglutination. Initially, both the direct agglutination and anti-globulin tests were used in all examinations but the former test was discontinued as the indirect anti-globulin test consistently gave the more reliable results.

When the agglutination tests were performed in a medium of bovine albumin (Diamond and Denton, 1945), a 2 per cent suspension of washed red cells was centrifuged, the supernatant saline replaced by an equal volume of 30 per cent bovine albumin solution (Armour & Company Ltd.) and the cells resuspended with a pipette. One drop of this suspension was added to one drop of serum of appropriate dilution and the mixture then incubated at 37°C. for 1 hr. Thereafter, the tubes were centrifuged at 3000 r.p.m. for 30 sec. and the deposited cells examined microscopically for agglutination.

Absorption of sera. The cells were washed three times in saline and centrifuged at 3000 r.p.m. for 10 min. The required volume of packed cells was then placed in each of a series of tubes containing saline. These tubes were then centrifuged as before and the supernatant fluid was removed just before the cells were used for absorption. The serum to be absorbed was added to the cells in equal volume and the two were mixed. After being incubated for 10 min. at 37°C. and centrifuged, the serum was removed and again absorbed with another volume of packed cells. Usually, four absorptions were performed in this way but if the absorbed serum still reacted against cells similar to those used for absorption, then the process was continued until all the antibody had been removed.

RESULTS

Separation of the Constituent Iso-antibodies in Sera from Cases of Haemolytic Disease

To avoid complication due to anti-A, the first sera analysed were from pigs of blood group A. Later, however, sera from group O pigs were also examined, as it was possible to inhibit the anti-A with an equal volume of a 1:5 dilution of boiled human saliva from a group A secretor. A serum inhibited in this way was then treated as if it were from a group A pig. To illustrate the general method, the analysis of one serum (sow 1) will be described in detail.

Analysis of Iso-antibodies in the serum of sow 1. Sow 1 was a group A pig. The serum, diluted 1:10, was first tested (I.S.T.) against the red cells of fifteen pigs comprising nine panel pigs and six from the slaughterhouse. All the cells gave a positive reaction. To see whether more than one iso-antibody was involved, aliquots of the serum were absorbed independently with the cells of ten of

these pigs. The absorbed samples, diluted 1:10, were then tested again against a panel of cells including those used for the absorption. A pattern was discernible which became more evident on re-arrangement (Table 1). Such a pattern suggested that antibodies of three specificities might be present, and these were called anti-1, anti-2 and anti-3.

The incompleteness of the series of tests shown in Table 1 for the serum sample* [abs. cells† P. 76] was due to the slaughterhouse bloods being no longer available. Further tests with cells from the panel, however, were consistent with the absorbed serum containing a single antibody, namely anti-3. Large samples of these three testing reagents were then prepared by absorbing the serum of sow 1 with cells from the pigs shown by the original absorbed sera to possess two out of the three antigens.

Tests for purity. To see if the prepared testing reagents anti-1, anti-2 and anti-3 each contained antibodies of a single specificity, nine aliquots of each reagent were re-absorbed with the cells of nine pigs with which they reacted. Each absorption removed the antibody for the other previously-reacting cells. Absorptions were also made with cells that did not react with the serum to show that the loss of antibody was specific. To increase the likelihood of separating antibodies to closely-linked antigens, the cells used in these purity tests were taken from unrelated animals that were, as far as possible, also of different breeds. Fresh samples of the testing reagents were tested for purity against a standard blood panel from ten pigs and these new reagents were not used for routine grouping of cells unless their reactions were identical with those obtained previously.

Separation of the Iso-antibodies in Further Serum Samples

The analysis of the serum from sow 1 had resulted in the definition of three red cell antigens. With the next serum, therefore, the donor pig's cells were first grouped for these three antigens and, subsequently, this grouping with as many reagents as were available preceded each analysis. It was thus possible to predict with increasing facility which antibodies might or might not be present in the serum to be examined. A brief description follows of the analyses, in the order that they were performed, to show how the eleven antigens were characterized. Some information on the serological properties of the different antigen-antibody systems is given in a separate section.

Analysis of Serum JB 146. With the existing testing reagents, the blood groups of this pig were (A 1 — 2 — 3 +). The serum, diluted 1:10, gave positive reactions (I.S.T.) with the cells of sixteen out

* To prevent confusion in the text, the complete description of serum samples will be given in square brackets. Note. abs. = absorbed.

† The identification prefixes P. and S. refer to panel and slaughterhouse pigs respectively.

TABLE 1

REACTIONS OF SERUM SOW 1

Serum Sow 1 absorbed by cells:	*Test cells*															*'Provisional naming of antibodies*
	S.10	*S.11*	*P.73*	*P.F10*	*P.76*	*S.7*	*S.9*	*S.12*	*P.F15*	*P.D42*	*S.8*	*P.60*	*P.37*	*P.F16*	*P.D41*	
S.10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S.11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S.8	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	anti-1
S.7	+	+	+	+	+	—	—	—	—	—	+	+	—	—	—	anti-2
S.9	+	+	+	+	+	—	—	—	—	—	+	+	—	—	—	anti-2
S.12	+	+	+	+	+	—	—	—	—	—	+	+	—	—	—	anti-2
P.76			+	+	—				+	+		—	+	+	+	anti-3
P.37	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	anti-1 & -2
P.F16	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	anti-1 & -2
P.60	+	+	+	+	+	+	+	+	+	+	+	—	+	+	+	anti-1 & -3
Unabsorbed	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	anti-1, -2 & -3
Antigens postulated	1, 2, 3	1, 2, 3	1, 2, 3	1, 2, 3	1, 2	1, 3	1, 3	1, 3	1, 3	1, 3	2, 3	2	3	3	3	

of seventeen pigs comprising ten panel pigs and seven pigs from the slaughterhouse. Aliquots of the serum were each absorbed with positively-reacting cells of one pig. After diluting to 1:10, the sera were re-tested against a number of the cells that had been used originally (Table 2).

At least four iso-antibodies appeared to be present in this serum. Serum [JB 146 abs. cells S.60] and serum [JB 146 abs. cells P.F13] contained an antibody to be called anti-4. Sera [JB 146 abs. cells S.56, S. 58, S.59 or S.61] must all have contained anti-4, together with an antibody to be called anti-5. The reactions of serum [JB 146 abs. cells P.F10] were due to an antibody to be called anti-6. That still another antibody was present in this serum was shown by the fact that none of the absorbed serum samples reacted with the cells of pig P.F16, despite the fact that these cells reacted with the unabsorbed serum. The antibody in question, which has not yet been separated, could not have been anti-1 or anti-2, as pig P.F16 lacked these antigens. Nor could it have been anti-3, as the absorption with cells S.56, S.59 and S.60 would have left this particular antibody in.

Tests for purity. Samples of serum anti-4 were absorbed respectively with eleven samples of positively-reacting cells and in each case no antibody reacting with any of these cells was left in. Hence the serum was considered to contain only anti-4.

Concerning the serum containing anti-4 and anti-5, in the initial analysis (Table 2) anti-5 was not separated from anti-4 and all subsequent attempts to obtain anti-5 free of anti-4 have been unsuccessful. All absorptions of this serum with pig cells reacting with anti-4, that is (4+) cells, have removed all the antibodies in the serum. As no animal with (4 + 5 −) cells has yet been found, it has not been possible to produce a pure anti-5 testing reagent. Absorptions with cells possessing antigen 5, but not antigen 4, left in a specific anti-4, but removed the antibody for all (4 − 5 +) cells. Although this serum contained two distinct antibodies, namely anti-4 and anti-5, it could, in conjunction with the standard anti-4 serum, be used for grouping. Doubt as to the presence of antigen 5 arises only when a blood also reacts positively with anti-4. As the frequency of antigen 4 has been found to be considerably lower than that of antigen 5 in the breeds of pigs so far studied, there are likely to be few blood samples where the constitution would need to be checked by an absorption test.

Samples of serum anti-6 were absorbed respectively with the cells of twelve positively-reacting animals and then tested back against the same panel of cells. Each of the absorptions removed the antibody for all the other cells, suggesting that only one antibody was involved.

Analysis of Serum A202. With the existing reagents, the blood groups of this pig were (A 1+ 2− 3− 4− 5− 6+). At a dilution

TABLE 2

REACTIONS OF SERUM JB 146

Reactions of serum JB 146 before and after selective absorptions on a panel of pig red cells.
(Results after rearrangement.)

Serum JB 146 absorbed by cells:	*Test cells*											*Provisional naming of antibodies*
	P.F10	*S.55*	*S.57*	*S.60*	*P.F13*	*P.F15*	*S.56*	*S.58*	*S.59*	*S.61*	*P.F16*	*P.B2*	
S.55	—	—	—	—	—	—	—	—	—	—	—	—	
S.57	—	—	—	—	—	—	—	—	—	—	—	—	
S.60	+	+	+	—	—	—	—	—	—	—	—	—	anti-4
P.F13	+	+	+	—	—	—	—	—	—	—	—	—	anti-4
S.56	+	+	+	+	+	+	—	—	—	—	—	—	anti-4 & -5
S.58	+	+	+	+	+	+	—	—	—	—	—	—	anti-4 & -5
S.59	+	+	+	+	+	+	—	—	—	—	—	—	anti-4 & -5
S.61	+	+	+	+	+	+	—	—	—	—	—	—	anti-4 & -5
P.F10	—	+	+	+	+	+	+	+	+	+	—	+	anti-6
Unabsorbed	+	+	+	+	+	+	+	+	+	+	+	+	anti-4, -5 & -6, plus another
Antigens known to be present on the cells	1, 2, 3	1, 3	1, 3	1, 2	1, 2, 3	1, 3	2	1, 2, 3	2	2, 3	3	3	
Additional antigens postulated	4, 5	4, 5, 6	4, 5, 6	5, 6	5, 6	5, 6	6	6	6	6	U	6	

U = Undesignated antigen.

of 1:10 the serum reacted positively (I.S.T.) with each of seventeen blood samples comprising nine from panel pigs and eight from the slaughterhouse. Aliquots of the serum were each absorbed with the cells of one animal of known blood-group structure and then tested (I.S.T.) against a panel of pig bloods (Table 3). These results suggested that at least four specific antibodies were present.

The serum [abs. cell S.82], tested against ten bloods, gave results comparable with anti-4; that is, it reacted only with the cells of pig P.F10. The reactions of sera [abs. cells S.84a or cells S.87] were those expected of anti-3, but anti-4 must also have been present as the cells S.84a and S.87 lacked antigen 4. Sera [abs. cells S.78 or S.79], besides possessing anti-4, contained a further antibody, not previously characterized. This was called anti-7. That yet another antibody, other than anti-3, anti-4 and anti-7, was present in serum A202 was shown by the fact that the cells of P.A318 and P.76 reacted with the serum when unabsorbed, but failed to react with any of the absorbed samples shown in Table 4. In an attempt to isolate this antibody, aliquots of serum A202 were absorbed with the cells of thirty-five pigs (Essex, Wessex, Large White and crossbred). In every case, however, the absorbing cells removed the unknown antibody.

Tests for purity. It had been deduced that the anti-7 reagents [serum A202 abs. cells S.78 or S.79] also contained anti-4. This was not particularly inconvenient, however, as the frequency of antigen 4 is low in the Large White, Essex and Wessex breeds, with which we were most concerned. Nevertheless, a pig was found eventually (P.F10, piglet 2) whose cells possessed antigens 3 and 4 but lacked antigen 7 and, using these cells for absorption, an anti-7 reagent, free of anti-4, was produced. However, although the usual purity tests indicated that this serum, which is now the standard anti-7 testing reagent, contained one specific antibody it has behaved irregularly, giving reactions of markedly different strengths with different positively-reacting cells. This has made the evaluation of results difficult and, consequently, the reagent is not wholly satisfactory.

Analysis of Serum P175. The blood groups of this animal were (A 1− 2+ 3+ 4− 5− 6+ 7−) and hence, of the known antibodies, it could have produced anti-1, anti-4, anti-5 and anti-7.

At a dilution of 1:10, the serum reacted strongly by direct agglutination with ten out of twelve bloods, and a reaction could be shown with eleven out of the twelve by the use of the anti-globulin sensitization test. Aliquots of the serum were then absorbed with the cells of individual pigs against which the serum had reacted and the reactions of the absorbed sera are shown in Table 4. The interactions observed were consistent with the presence of four antibodies: anti-1, anti-4 (or anti-7), anti-5 and another antibody, provisionally called anti-8. Further absorptions and tests

TABLE 3

REACTIONS OF SERUM A202

Reactions of serum A202 before and after selective absorptions on a panel of pig red cells.

(Results after rearrangement.)

Serum A202 absorbed by cells:	*Test cells*																	*Provisional naming of antibodies*
	P. F10	*S. 86*	*S. 88*	*P. D41*	*P. D42*	*P. F16*	*P. BB*	*S. 78*	*S. 79*	*S. 85*	*S. 89*	*S. 82*	*S. 84a*	*S. 87*	*P. A318*	*P. 76*	*P. 60*	
S.82 ..	+		—					—	—	—	—	—	—	—		—		anti-4
S.84a ..	+	+	+	+	+	+	+			+	+		—	—	—	—	—	anti-3 & anti-4
S.87 ..	+	+	+	+	+	+	+			+	+		—	—	—	—	—	
S.78 ..	+	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	anti-4 & anti-7
S.79 ..	+	—	—	—	—	—	—	—	—	+	+	+	+	+	—	—	—	
P.76 ..	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	anti-3, -4 & -7
Unabsorbed ..	+			+	+	+		+	+			+			+	+		anti-3, -4 & -7, plus another
Antigens known to be present on the cells	1, 2, 3, 4, 5	1, 3, 6	3, 6	3	1, 3	3	1, 3, 6	3, 5, 6	1, 3, 6	3, 6	3, 6	1, 3, 5, 6	6	2, 6	6	1, 2, 6	2, 6	
Additional antigens postulated	7*									7	7	7	7	7	U	U		

* Shown by absorption. U = Undesignated antigen.

TABLE 4

REACTIONS OF SERUM P175

Reactions of serum P175 before and after selective absorptions on a panel of pig red cells.
(Results after rearrangement.)

Serum P175 absorbed by cells:	*Test cells*										*Provisional naming of antibodies*
	P.F10	*P.G114*	*P.A318*	*P.G5*	*P.F12*	*P.G4*	*P.G49*	*P.73*	*P.134*	*P.F14*	
P.F15	+	—	—	—	—	—	—	—	—	—	anti-4
P.G114	+	—	—	—	—	—	—	—	—	—	anti-4
P.G4	+	+	—	—	—	—	—	—	—	—	anti-4 & -5
P.G49	+	+	—	—	—	—	—	—	—	—	anti-4 & -5
P.F12	+	+	—	—	—	—	—	—	—	—	anti-4 & -5
P.134	+	+	+	+	+	+	+	—	—	—	anti-4, -5 & -8
P.73	+	+	+	+	+	+	+	—	—	—	anti-4, -5 & -8
P.F14	+	+	+	+	+	+	+	—	—	—	anti-4, -5 & -8
P.G5	+	+	—	—	+	+	+	+	+	+	anti-1, -4 & -5
Unabsorbed ..	+	+	+	+	+	+	+	+	+	+	anti-1, -4, -5, & -8
Antigens known to be present on the cells	1, 2, 3, 4, 5, 7	1, 3, 5, 6	6	3, 6	1, 3, 6	1, 3, 6	1, 3, 6	1, 2 3, 6	1, 6	1, 3, 6	
Further antigens postulated	8	8	8	8	8	8	8				

confirmed the presence of anti-1 and anti-5. While the evidence in Table 4 suggested the presence of anti-4 or anti-7, or both of these antibodies, as the corresponding antigen in both cases was present only on the test cells of one pig, namely P.F10. Subsequently, a (4— 7+) blood was found and, as this failed to react with the sera [abs. cells P.F15 or P.G114], it was concluded that it was anti-4, and not anti-7, which was present in the serum. The presence of anti-8 was evident from the reactions of sera [abs. cells P. 134, P.73 or P.F14] but anti-4 and anti-5 must have been present also in these samples, as the cells used for absorption (P.134, P.73 and P.F14) did not possess either antigen 4 or 5. Subsequently, a sample of anti-8 was prepared, free of these contaminants, by absorbing with a mixture of cells that, in combination, possessed antigens 1, 4 and 5.

Tests for purity. Samples of anti-8 were absorbed with the cells of ten pigs, eight of which possessed the corresponding antigen. The positively-reacting cells removed all antibody from the serum, thus confirming its single specificity.

Preparation of Three Further Testing Reagents

In order to obtain further reagents as rapidly as possible, two more sera were partially analysed, attention being concentrated on any unknown antibodies.

Serum D42 yielded one such antibody, anti-9, contaminated with anti-4 and anti-5. Because of the high frequency of antigen 9 it has not yet been possible to purify this reagent further, but used in conjunction with reagents anti-4 and anti-5 it is a reliable grouping serum. Furthermore, its usefulness is enhanced by the infrequent occurrence of antigens 4 and 5 in most of the breeds studied. Serum ON yielded two further antibodies, anti-10 and anti-11, as well as anti-8.

Purity tests on these three reagents indicate that there are no contaminants other than anti-4 and anti-5 in reagent anti-9 and that sera anti-10 and anti-11 are of single specificity.

Eleven testing reagents have been prepared by analysis and absorption of serum samples taken from sows having litters affected with haemolytic disease. These reagents each contain either a single antibody or a known mixture of antibodies and they are capable of defining eleven red cell antigens in the pig. Samples of each have been freeze-dried for future reference.

Recognition of Antigen-Antibody Systems 1 to 8 by Five Serological Techniques

In order to find the optimal method for demonstrating the first eight systems characterized, comparative titrations were made using five routine blood-grouping techniques. It was hoped that this information would also help other workers to identify the systems concerned. The tests for each system were made, wherever possible,

with the iso-antibody prepared from three different sera and with red cells from the blood of two different animals. It is probable, therefore, that the results truly reflect the properties of the systems under test, rather than any variations of particular serum samples. The techniques that were compared were direct agglutination (A and B), direct agglutination in an albumin medium (B); direct agglutination using trypsin-treated cells (B), and the anti-globulin sensitization test (A). (A) indicates that the results were read after sedimentation and (B) after being centrifuged for 30 sec. The serum titres obtained with each system are shown in Table 5.

With antigen-antibody systems 1 and 2 the interaction was well demonstrated by direct agglutination, whereas the only satisfactory method for demonstrating systems 3 and 6 was the anti-globulin test. The latter test was also the optimal method for systems 4, 5 and 8. Previous treatment of the cells with trypsin enhanced the direct agglutination with antigens 4, 5 and 8.

The Antigen-Antibody Systems in Cases of Haemolytic Disease of the New-born

In our cases of haemolytic disease, the serum iso-antibodies had seemingly been produced by injections of crystal violet swine fever vaccine. As this vaccine is prepared from the bulked blood of many pigs, and as two or more injections are nearly always necessary before the clinical disease ensues (Goodwin and Saison, 1957), it follows that a very wide range of antigens, about which we could obtain no direct information, had been injected into the sows. But by choosing the sera to be analysed from sows that had clinically affected their litters, rather than from pigs that had been injected experimentally with blood of known groups, it was possible to return more readily to the question of the blood-group patterns involved in the disease in the field.

Initially, the prime object had been to isolate and purify testing sera, but as the work proceeded it was possible to begin the examination of cases of haemolytic disease. This was carried out mainly by blood-grouping the red cells of the sow and boar, a procedure that not only suggested those antibodies that could, theoretically, have given rise to the disease, but sometimes indicated the presence of further antigen-antibody systems. To see which of the known antibodies were actually present, pilot absorptions of aliquots of the serum were made with cells from either four or five animals selected for their blood groups. Then, by testing the absorbed samples on a panel of ten selected bloods a good indication was obtained of those antibodies of the known series that might have produced the disease.

Table 6 brings together all the blood-group data that is at present available for twenty field cases of haemolytic disease. In general, it will be seen that although the over-all antibody titre is always given, only with the earlier serum samples has there been

TABLE 5

COMPARISON OF FIVE SEROLOGICAL TECHNIQUES

The comparative ability of five serological techniques to demonstrate the antibody in each of eight different blood-group systems.

Reagents			*Antibody titre as shown by:*				
Antibody	*Serum*	*Cells*	*Agglutination*	*Agglutination (centrifuged)*	*Agglutination (albumin)*	*Agglutination (trypsin)*	*Anti-globulin test*
Anti-1	R4	76	(4)	8	8	8	16
		114	4	8	(4)	16	32
	D41	76	16	16	8	16	16
		114	8	16	16	32	32
	FD7	76	16	32	16	64	32
		114	16	32	32	32	64
Anti-2	CD115	73	32	64	16	64	16
		125	32	32	16	64	16
	FD7	73	4	8	4	8	8
		125	8	8	8	8	8
	Sow 1	73	(4)	4	4	4	8
		125	4	8	4	8	8
Anti-3	R4	F10	0	(4)	8	4	128
		114	0	0	(4)	4	128
	K	F10	0	0	(4)	(4)	64
		114	0	0	(4)	(4)	32
	Sow 1	F10	0	(4)	8	8	64
		114	0	0	(4)	(4)	128
Anti-4	JB146	HT	0	0	(2)	2	16
		F10, p.8	0	0	2	2	16
	P175	HT	0	0	(2)	8	8
		F10, p.8	0	0	(2)	8	8
	D42	HT	2	4	(2)	4	32
		F10, p.8	0	0	(2)	2	32
Anti-5	JB146	F15	(2)	(2)	(2)	4	32
		G114	(2)	(2)	(2)	2	32
	P175	F15	(2)	(2)	4	32	64
		G114	(2)	(2)	2	32	32
	D42	F15	(2)	(2)	(2)	8	64
		G114	(2)	(2)	(2)	8	32

Table 5—*Continued*

Reagents			*Antibody titre as shown by:*				
Antibody	*Serum*	*Cells*	*Agglutination*	*Agglutination (centrifuged)*	*Agglutination (albumin)*	*Agglutination (trypsin)*	*Anti-globulin test*
Anti-6	K	73	0	0	0	(4)	128
		114	0	0	0	(4)	128
	CD115	73	0	0	0	(4)	128
		114	0	0	0	(4)	256
	JB146	73	0	0	0	(4)	64
		114	0	0	0	(4)	64
Anti-7	A202	GR1	4	4	16	(4)	16
		F10, p.1	8	8	16	4	16
Anti-8	ON	132	8	16	64	256	256
		F12	8	16	64	128	256
	CD115	132	4	4	16	16	64
		F12	4	8	16	16	64
	P175	132	8	8	16	64	64
		F12	8	16	16	64	256

The lowest dilution tested was 1 in 2 with anti-4 and anti-5 and 1 in 4 with the other sera. At these dilutions, 0 = no agglutination, and ( ) = weak agglutination.

time so far to determine some of the constituent titres. Conversely, with the later cases appearing lower in the table, more is known of the blood groups of the sow and boar. It would appear that haemolytic disease of the new-born pig may be caused by antibodies of several specificities and although anti-3, anti-6 and anti-8 are the most conspicuous, they are not the only possibilities from among the known series. Furthermore, in several of the sera there are undesignated antibodies, which in some instances are known to be at a high titre.

Although some of the blood-group patterns underlying the disease are now emerging, a number of important questions remain unanswered. In particular, information is required on the broad epidemiological factors that restrict the disease to certain breed matings. These factors are now being investigated, however, and the first stage of this work should be completed shortly.

CONCLUSIONS

The sera from six sows that affected their litters with haemolytic disease have been analysed to produce specific testing reagents. With these reagents, eleven red cell antigens of the pig have been characterized.

TABLE

BLOOD-GROUP PATTERNS

The blood-group structures and specific

	Blood-group antigens of sow and boar, and																				
Sow	*A*			*1*			*2*			*3*			*4*			*5*					
Sow 1	+				32			16			512										
JB146	+		—	—	4	—	—	4	—	+		+	—	16	—	—	128	—			
A202	+			+		+	—		—	—	512	+	—	32		—					
P175	+		+	—	128	+	+		—	+		+	—	8	—	—	32	—			
D41	—			—	32		—	0		+			—	0		—	0				
D42	—			+			—			+			—	256		—	16				
R4	+			—	64		+			—	128		—	0		+					
CD115	—		—	+		+	—	64	—	+		+	—		—						—
ON	—		—	—		—	—		—	+		+	+		—	+					—
K	+		+	—	16	—	+		—	—	128	+	—	0	—	—	0	—			
DEB 1	—	>10	+	—		—	+		—	—		+	+		—	+		—			
DEB 2	—	>10	+	—	0	—	—	0	—	+		+	+		—	+		—			
H 103	+			—			+			+			—			—					
H 105	+			—			—			+			—			—					
CO	+		+	+		+	—		—	+		+	—		—	—					+
EL 56	+		—	—		—	—		+	+		+	—		—	—					—
CLL	+		—	+		+	—		—	+		+	—		—	—					+
MDA	—	>10	—	—		—	+		+	+		+	—		—						—
RER	—		—	—		—	—		—	+		+	—		—	—					—
GEY	+		+	—		+	—		—	+		+	—		—	—					

Note. 1. With each blood-group column, the symbols on the left refer to the sow and those on the right to the boar. The centre figures denote the titre of the specific antibody in the sow's serum.

2. All the sows listed had lost one or more piglets with haemolytic disease.

6

IN HAEMOLYTIC DISEASE

iso-antibodies in cases of haemolytic disease.

individual iso-antibody titres where known														
6			*7*			*8*			*9*			*Over-all titre*	*Antibodies possibly responsible*	*Sow*
—		+	—		—	+						2048	anti-3	Sow 1
—	128	+	—		—	+						512	*anti-6	JB146
+		+	—	32	—	+						1024	*anti-3	A202
+		—	—		—		128					512	*anti-1, anti-8	P175
—	64		—	0		+						1024	*	D41
—			—			+				256		512	‡anti-9	D42
—	32		+			+			+			>1024	*anti-3	R4
—	256	+	—		—	—	128	+				2048	anti-6, anti-8	CD115
+		+	—		—	—	1024	+	+		+	2048	*anti-8	ON
—	256	+	—	0	+		0					1024	*anti-3, anti-6	K
+		+		0	—	—		+	+		—	>1024	*	DEB 1
+		+	—	0	—	—	>10	+	+		—	>1024	*	DEB 2
+						—			+			>512		H 103
+			—			—			+			>512		H 105
—		+			—	+		—	+		+	>512		CO
+		+ +	—		— —	+		+ +	+		+ +	64	†	EL 56
+		+	—		—	—		+	+		+	4096		CLL
+		+ +		0	— —	—		+ +				2048	†	MDA
+		+	—			—		+	+		+	64	anti-8	RER
—		—	—		—	—		+	+		+	1000	*anti-8	GEY

* In these instances the serum also contained an undesignated antibody which, with sows D41 and GEY, was at a titre of 256.

† As there were two possible sires, the blood-groups of both are given.

‡ It is unlikely that anti-4 contributed to the disease, because the sire was a Large White and the incidence of antigen 4 among Large White pigs is very low.

0 = Antibody known to be absent.

Anti-4, anti-5 and anti-8 are best demonstrated by the anti-globulin sensitization test, and the latter test is the only satisfactory method for detecting anti-3 and anti-6. Anti-1 and anti-2, however, can be well demonstrated by direct agglutination.

Some progress has been made in enumerating the iso-antibodies that could have been concerned in twenty field cases of haemolytic disease. To date, those most commonly encountered have been anti-3, anti-6 and anti-8.

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THE METABOLISM OF KETONE BODIES IN THE RUMINANT

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INTRODUCTION

Until fairly recently most of the investigations carried out on the administration of the various ketone bodies have been conducted with a view to determining the part they play in diabetes and diabetic coma in man, dog, etc. or to elucidating the processes of fat metabolism in the animal body. In none of these investigations has the ruminant been used as the experimental animal. During the past few years, however, increasing interest has been shown in Ruminant Ketosis and various experiments involving the administration of ketone bodies to ruminants have been carried out. Nevertheless, in very few of these have the ketones been estimated separately, and in none has the part played by isopropanol been studied. As the metabolism of ketones in ruminants could differ from that in single-stomached animals, it was thought advisable to investigate the changes that occur in the levels of ketone bodies in the blood and rumen contents when individual ketone bodies are administered to cattle and to sheep. It was hoped that the results might not only throw some light on the metabolism of the ketone bodies but possibly indicate the part played by them in ruminant ketosis.

METHODS

The administration experiments covered a period of either four hours or four to five days during which time the animals were fed and watered as usual. The sheep had rumen fistulae and were kept in indoor pens; the cows were normal animals in a dairy herd and were kept in their stalls for the duration of the experiment only. Except where otherwise indicated in the text four animals were used for each experiment and the results averaged.

Access to the rumen in the case of the sheep was obtained by means of a rumen cannula with a screw cap. A long stomach tube was employed for cows both for the removal of rumen contents and for the introduction of substances into the rumen. The ketones were introduced into the rumen dissolved in warm water, the volumes used being 500 to 1,000 ml. for the cow and 100 to 200 ml. for the sheep. For intravenous injection the ketone solutions were made up in physiological saline at body temperature, the total volume injected being about 200 ml. for the cows and 20 ml. for the sheep. The solutions were injected slowly into the jugular vein in every case.

Samples of blood and rumen liquor were taken immediately before administration and at definite times thereafter, e.g. 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours in the case of single-dose experi-

TABLE 1

KETONE BODY LEVELS IN RUMEN CONTENTS AND BLOOD FOLLOWING ADMINSTRATION OF A SINGLE DOSE INTO THE RUMEN

Expressed as mg. acetone/100 ml.

Substance administered	*Ketone body*	*Time after administration*											
		Rumen liquor						*Blood*					
		0	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*	*0*	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*
COWS													
Sod. β-hydroxybutyrate 100 g.	Acetone	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	Isopropanol	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	Acetoacetic Acid	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	β-hydroxybutyric Acid	2·8	124·1	41·7	55·7	31·5	45·9	7·1	6·0	8·3	7·2	4·4	4·1
Methyl Acetoacetate 100 ml.	Acetone	0·0	0·6	0·2	0·2	0·0	0·0	0·0	0·0	0·2	0·2	0·1	0·0
	Isopropanol	0·0	0·4	1·3	0·3	0·2	0·2	0·0	0·0	0·1	0·0	0·0	0·0
	Acetoacetic Acid	0·0	39·1	22·0	6·1	2·6	0·6	0·0	2·5	1·7	1·1	0·3	0·4
	β-hydroxybutyric Acid	6·7	4·1	0·8	7·2	8·8	6·3	3·7	14·6	16·4	16·4	14·4	11·8
Acetone 100 ml.	Acetone	0·0	100·5	42·2	31·2	18·8	12·4	0·0	4·8	10·6	12·0	14·7	17·2
	Isopropanol	0·0	3·8	5·8	7·0	10·2	7·9	0·0	0·0	0·4	0·6	0·9	0·6
	Acetoactic Acid	0·0	4·2	3·6	1·4	1·9	1·0	0·0	1·4	1·7	2·8	1·7	4·7
	β-hydroxybutyric Acid	3·4	14·2	12·5	8·9	10·1	8·3	5·8	7·1	5·3	6·1	8·9	9·8
Isopropanol 250 ml.	Acetone	0·0	4·0	8·0	11·7	21·0	20·3	0·0	4·0	6·7	11·7	23·5	24·9
	Isopropanol	0·0	166·1	65·6	52·9	45·8	23·2	0·0	9·4	14·0	15·8	10·5	8·8
	Acetoacetic Acid	0·0	2·6	4·4	1·2	1·1	3·2	0·0	1·0	1·5	2·0	4·1	4·8
	β-hydroxybutyric Acid	2·8	295 6	61·7	12·1	3·1	14·0	5·4	18·0	34·0	22·4	8·4	11·0

SHEEP													
Sod. β-hydroxybutyrate 10 g.	Acetone	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	Isopropanol	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	Acetoacetic Acid	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	β-hydroxybutyric Acid	0·0	32·6	28·9	20·0	17·8	12·3	0·0	3·8	0·7	1·6	2·8	1·5
Methyl Acetoacetate 4 ml.	Acetone	0·0	1·6	0·1	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0
	Isopropanol	0·0	0·0	0·6	0·9	0·9	0·7	0·0	0·0	0·0	0·1	0·2	0·4
	Acetoacetic Acid	0·0	66·6	15·6	7·2	2·1	0·0	0·0	0·0	0·0	0·3	0·0	0·0
	β-hydroxybutyric Acid	1·3	2·2	3·6	5·4	2·0	1·5	7·3	9·9	11·1	4·9	7·5	5·9
Acetone 4 ml.	Acetone	0·0	67·1	24·6	17·0	9·1	5·6	0·0	3·0	4·7	3·8	4·3	4·3
	Isopropanol	0·0	0·9	1·8	4·6	2·1	1·7	0·0	0·0	0·2	0·4	0·5	0·5
	Acetoacetic Acid	0·0	1·6	1·4	1·6	1·7	0·9	0·0	1·2	1·4	1·0	1·1	1·1
	β-hydroxybutyric Acid	2·8	7·7	3·7	3·1	3·7	4·3	6·8	7·4	5·7	7·0	5·7	5·5
Isopropanol 15 ml.	Acetone	0·0	0·9	2·1	3·7	6·8	9·2	0·0	2·1	4·6	8·1	9·9	12·2
	Isopropanol	0·0	190·0	111·5	66·5	25·9	10·9	0·0	5·4	6·4	2·5	2·3	3·2
	Acetoacetic Acid	0·0	0·5	1·3	0·6	1·5	1·0	0·0	0·6	1·8	1·6	2·9	1·7
	β-hydroxybutyric Acid	9·9	197·7	10·7	16·4	26·6	12·5	17·7	8·9	6·1	12·1	10·3	3·8

ments. Usually about 50 ml. blood was taken from the mammary vein of the cow, and about 25 ml. from the unused jugular vein of the sheep. In some cases milk and urine samples were also taken from the cows but, since the cows were not milked out at each sampling, the results for milk samples are cumulative over the period studied.

The ketone bodies, acetone, acetoacetic acid, β-hydroxybutyric acid and isopropanol were estimated as previously described (Thin and Robertson, 1952).

Normal animals were used in all experiments and β-hydroxybutyric acid was the only ketone body detected in the blood and rumen contents prior to administration.

RESULTS

Administration of a Single Dose into the Rumen

Changes in the concentration of ketone bodies in rumen contents and blood of cows and sheep subsequent to the administration of a single dose of an individual ketone body into the rumen are shown in Table 1.

Following the administration of sodium β-hydroxybutyrate, only that same ketone body was found and, although the initial high level in the rumen fell off rapidly, the blood levels did not appear to alter significantly and never rose above the normal range of 0 to 10 mg/100 ml. expressed as acetone. Methyl acetoacetate gave a broadly similar picture in that only traces of acetone or isopropanol appeared in the blood, while the increases in blood acetoacetate and β-hydroxybutyrate levels which occurred were relatively slight in cows and absent in sheep. In rumen contents the acetoacetate levels declined rapidly without any marked rise in acetone, isopropanol or β-hydroxybutyrate levels, the maximum rise noted being 1·6 mg. of acetone per 100 ml. rumen contents for sheep.

The responses with acetone and isopropanol contrasted with those for β-hydroxybutyrate and acetoacetate in that there was a substantial response in the blood level of the substance administered. Further, there was evidence of a rapid interconversion of acetone and isopropanol so that four hours after administration somewhat similar concentrations of isopropanol and acetone were present in rumen contents, no matter which substance had been given. The conversion of isopropanol to acetone seemed particularly marked in that four hours after isopropanol administration the blood acetone level was considerably higher than that of isopropanol and of the rumen acetone. Another interesting feature was the apparent rapid formation of large amounts of β-hydroxybutyrate from isopropanol in the rumen.

The effects of rumenal acetone and isopropanol administration on the blood levels of acetoacetate were not large, but were appreciably greater than those induced by the administration of either methyl acetoacetate or sodium β-hydroxybutyrate. There was a

TABLE 2

KETONE BODY LEVELS IN MILK AND URINE OF COWS FOLLOWING ADMINISTRATION OF A SINGLE DOSE INTO THE RUMEN

Expressed as mg. acetone/100 ml.

Substance administered	*Ketone body*	*Time after administration*									
		Milk						*Urine*			
		0	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*	*0*	*1 hr.*	*2 hr.*	*4 hr.*
Methyl Acetoacetate 100 ml.	Acetone	0·0	0·0	0·0	0·0	0·0	0·0	0·0		3·4	0·0
	Isopropanol	0·0	0·0	0·0	0·0	0·0	0·0	0·0		0·5	0·0
	Acetoacetic Acid	0·0	2·8	0·6	0·9	0·9	0·0	0·0		15·7	1·0
	β-hydroxybutyric Acid	4·6	7·7	5·6	9·1	10·8	12·3	6·2		16·6	19·9
Acetone 100 ml.	Acetone	0·0	1·1	6·8	12·4	6·1	13·5	0·0	10·8		17·1
	Isopropanol	0·0	0·0	0·0	0·2	0·6	0·8	0·0	0·9		1·4
	Acetoacetic Acid	0·0	2·0	0·3	3·9	6·5	3·4	0·0	1·0		0·7
	β-hydroxybutyric Acid	4·6	30·8	29·2	21·3	7·2	3·2	8·0	16·8		10·4
Isopropanol 250 ml.	Acetone	0·0	0·0	2·7	12·2	20·3	38·3	0·0			29·7
	Isopropanol	0·0	4·0	12·2	20·0	18·0	8·2	0·0			18·0
	Acetoacetic Acid	0·0	0·0	2·5	0·8	2·5	1·1	0·0			1·1
	β-hydroxybutyric Acid	8·0	29·2	24·3	43·5	19·7	5·3	10·1			144·3

TABLE 3

KETONE BODY LEVELS IN BLOOD AND RUMEN OF COWS AND SHEEP FOLLOWING THE ADMINISTRATION OF A SINGLE DOSE INTRAVENOUSLY

Expressed as mg. acetone/100 ml.

Substance administered	*Ketone body*	*Rumen*						*Blood*					
		0	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*	*0*	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*
Cows													
Sod. β-hydroxybutyrate	Acetone Isopropanol Acetoacetic Acid	} Amounts too small to estimate						} Amounts too small to estimate					
25 g.	β-hydroxybutyric Acid	2·5	0·0	0·0	0·4	0·0	0·0	6·6	3·4	3·4	4·3	4·6	3·1
40 g.	do	2·0	1·7	1·9				1·6	5·3	3·3			
Methyl Acetoacetete 30 ml.	Acetone	0·0	0·2	} Amounts too small to estimate				0·0	0·3	} Amounts too small to estimate			
	Isopropanol	0·0	0·1					0·0	0·1				
	Acetoacetic Acid	0·0	0·6					0·0	1·1				
	β-hydroxybutyric Acid	2·5	1·3					3·8	2·7				
Acetone 75 ml.	Acetone	0·0	1·4	4·5	4·7	5·8	5·4	0·0	12·5	10·5	10·4	10·1	9·6
	Isopropanol	0·0	1·1	2·9	3·7	4·0	5·0	0·0	0·1	0·2	0·3	0·2	0·3
	Acetoacetic Acid	0·0	0·4	0·4	0·8	0·8	0·8	0·0	1·6	1·6	1·9	1·2	1·7
	β-hydroxybutyric Acid	1·2	5·7	5·9	4·3	2·7	4·4	5·6	7·4	8·1	7·6	9·4	6·4
Isopropanol 125 ml.	Acetone	0·0	1·4	2·2	3·7	6·6	4·4	0·0	2·0	6·5	9·1	13·5	10·1
	Isopropanol	0·0	8·1	5·3	7·9	5·3	8·4	0·0	18·0	10·1	7·1	2·8	1·2
	Acetoacetic Acid	0·0	0·9	0·5	1·1	1·1	1·0	0·0	1·4	0·7	2·2	0·9	2·4
	β-hydroxybutyric Acid	5·2	8·0	7·7	4·4	5·4	6·3	8·2	20·4	19·1	2·5	4·3	5·5

Sheep													
Sod. β-hydroxybutyrate 5 g.	Acetone	0·0	} Amounts too small to estimate					0·0	} Amounts too small to estimate				
	Isopropanol	0·0						0·0					
	Acetoacetic Acid	0·0						0·0					
	β-hydroxybutyric Acid	1·0	1·0	1·0	1·0	0·0	0·0	12·9	18·7	13·6	11·3	6·8	8·6
Methyl Acetoacetate 5 ml.	Acetone	0·0	0·0	} Amounts too small to estimate				0·0	1·4	} Amounts too small to estimate			
	Isopropanol	0·0	0·0					0·0	0·2				
	Acetoacetic Acid	0·0	0·3					0·0	2·2				
	β-hydroxybutyric Acid	1·3	1·0					2·4	8·3				
Acetone 4 ml.	Acetone	0·0	1·7	2·4	3·2	2·9	3·1	0·0	12·5	11·0	9·3	7·1	6·1
	Isopropanol	0·0	0·8	1·0	1·2	1·6	2·3	0·0	0·0	0·3	0·4	0·4	0·9
	Acetoacetic Acid	0·0	1·4	0·8	1·3	0·8	1·0	0·0	2·1	1·5	2·0	1·5	1·5
	β-hydroxybutyric Acid	3·4	2·7	5·7	3·2	3·6	3·3	9·3	2·5	4·3	4·5	6·3	3·6
Isopropanol 4 ml.	Acetone	0·0	1·1	1·3	1·6	3·0	4·2	0·0	3·2	4·4	5·9	6·8	8·1
	Isopropanol	0·0	1·4	4·8	4·4	5·1	2·3	0·0	7·1	5·9	4·4	2·9	1·2
	Acetoacetic Acid	0·0	0·4	0·9	0·9	0·7	0·8	0·0	0·8	1·0	2·3	2·5	1·7
	β-hydroxybutric Acid	1·3	5·8	7·0	10·4	7·5	7·5	9·3	30·0	26·7	16·5	5·0	9·8

strong smell of acetone in the breath for a few hours after the administration of both acetone and acetoacetate but no clinical signs of ketosis were observed.

In the case of cows, samples of milk and urine were taken at intervals and the results are shown in Table 2. In general, these results were consistent with the blood values. Acetone and isopropanol had greater effects than methyl acetoacetate; in milk, as in blood, the isopropanol-acetone equilibrium resulted eventually in a marked predominance of acetone, and the ruminal administration of acetone or of isopropanol appeared to be capable of giving rise to considerable amounts of β-hydroxybutyric acid in both urine and milk.

Administration of a Single Dose Intravenously

Results for the intravenous administration of a single dose of a ketone body in cattle and sheep are shown in Table 3 which gives average results from four animals of each species except in the case of acetoacetate where only two were used.

When 25 g. or 40 g. of sodium β-hydroxybutyrate were injected into cows, no other ketone bodies were found in either blood or rumen contents and the levels of β-hydroxybutyric acid did not alter appreciably. A similar lack of response followed the intravenous injection of 5 g. of sodium β-hydroxybutyrate in sheep. After most injections of methyl acetoacetate the animals suffered from dyspnoea and the breath smelt heavily of acetoacetate; 75 to 80 ml. of acetoacetate intravenously into cows acted as a temporary anaesthetic followed by laboured breathing and a great deal of coughing. Because of the risk of lung damage only 30 ml. were injected in subsequent experiments with cows and the amount was limited to 5 ml. in the case of sheep. In samples taken three and ten minutes after intravenous injections of methyl acetoacetate, acetoacetic acid was found only in small amounts in both blood and rumen with traces of acetone and isopropanol. There was little change in the levels of β-hydroxybutyric acid. At subsequent samplings the amounts of ketone bodies present were too small to estimate.

The effects of intravenous acetone and isopropanol were similar to those obtained with ruminal administration. Transference from blood to rumen readily occurred so that four hours after administration the concentration of the two substances taken together was approximately the same in the two systems. By then the rumen contents contained approximately equal concentrations of acetone and isopropanol, while in blood the ratio of acetone to isopropanol was of the order of 10 to 1, irrespective of which substance was administered. This suggests rapid conversion of isopropanol to acetone in the blood or other tissues, a possibility supported by the intra-rumen experiments. There was also evidence of the rapid

TABLE 4

KETONE BODY LEVELS IN MILK AND URINE OF COWS FOLLOWING ADMINISTRATION OF A SINGLE DOSE INTRAVENOUSLY

Expressed as mg. acetone/100 ml.

Substance administered	*Ketone body*	*Time after administration*										
		Milk						*Urine*				
		0	*10 mins.*	*30 mins.*	*1 hr.*	*2 hr.*	*4 hr.*	*0*	*10 mins.*	*1 hr.*	*2 hr.*	*4 hr.*
Methyl Acetoacetate 30 ml.	Acetone	0	1·4					0	3·7			
	Isopropanol	0	0					0	0			
	Acetoacetic Acid	0	1·1					0	30·3			
	β-hydroxybutyric Acid	8·5	12·3					12·6	54·3			
Acetone 75 ml.	Acetone	0	1·7	5·9	6·8	6·4	7·9	0		7·9		8·1
	Isopropanol	0	0	0	0	0	0·3	0		0·8		0·8
	Acetoacetic Acid	0	1·2	1·7	0·9	1·9	1·3	0		0·6		1·0
	β-hydroxybutyric Acid	9·7	7·4	4·9	7·4	7·2	7·9	4·2		7·6		6·6
Isopropanol 125 ml.	Acetone	0	3·4	4·3	7·0	8·6	15·5	0		7·7	11·7	14·9
	Isopropanol	0	9·6	8·9	7·9	6·5	2·0	0		15·0	7·9	4·4
	Acetoacetic Acid	0	0·6	0·3	1·6	2·2	0·7	0		0·1	1·0	0·5
	β-hydroxybutyric Acid	5·6	0·4	4·7	1·7	6·6	0·3	14·4		21·5	28·3	39·8

formation of β-hydroxybutyric acid from isopropanol, especially in the tissues.

Milk and urine samples taken from cows injected with acetone, isopropanol and methyl acetoacetate gave the results shown in Table 4. After the administration of acetone intravenously no isopropanol was found in the milk until four hours after injection and then only in trace amounts. The levels of acetone in milk were slightly lower than in the blood, while those of acetoacetic acid and of β-hydroxybutyric acid remained low and were similar to those of blood. In the urine the most prominent ketones were acetone and β-hydroxybutyric acid. Following the administration of isopropanol, acetone and acetoacetic acid were found in the milk in about the same proportions as in blood, whilst β-hydroxybutyric acid occurred at lower levels. In contrast with the effect of ruminal administration of acetone and isopropanol on the β-hydroxybutyric acid level of milk, intravenous injection of these two substances had negligible effects. In all urine samples taken after injection of isopropanol β-hydroxybutyric acid was the main excretory product. A short time after injection isopropanol was found in fairly high amounts, but later samples contained decreasing amounts of isopropanol and increasing amounts of acetone. With acetoacetate, β-hydroxybutyric acid was the main ketone excreted in the milk with traces of acetone and acetoacetic acid. Ten to fifteen minutes after the injection of acetoacetate, acetone was found in the urine with substantial amounts of acetoacetic acid and a greater amount of β-hydroxybutyric acid.

Repeated Administration

Owing to the mechanical difficulties involved where large experimental animals are concerned, the constant drip method of continuous administration was not utilised. Instead, the animals were given two or four injections per day, the number depending mainly on the tractability of the animal. Two animals were used in each experiment and the results averaged. Samples were taken daily just before the first injection of the day.

Repeated injections of acetone intravenously (Table 5). In the cow, as would be expected from the results of the previous experiments, there was a steady daily rise in the concentrations of acetone and acetoacetic acid in the blood. The concentration of β-hydroxybutyric acid rose over the period of experiment, while only traces of isopropanol were found. Acetone, acetoacetic acid and β-hydroxybutyric acid rose in the rumen contents, as in the blood, and there was a very much more pronounced rise in isopropanol. The levels of ketone bodies found in the milk were very similar to those in the blood. β-hydroxybutyric acid was the main excretory product in the urine until the third day when acetone and β-hydroxybutyric acid were found in about equal amounts. In general the response of the sheep was similar to that of the cow, though slightly higher

TABLE 5

KETONE BODY LEVELS FOLLOWING REPEATED INTRAVENOUS INJECTIONS OF ACETONE

Expressed as mg. acetone/100 ml.

		Days of Experiment										
		Cows					*Sheep*					
		0	*1*	*2*	*3*	*5*	*0*	*1*	*2*	*3*	*4*	*5*
	Acetone injected	2 × 50 ml.	2 × 75 ml.	2 × 75 ml.			4 × 2·5 ml.	4 × 2·5 ml.	4 × 5 ml.	4 × 5 ml.		
Rumen Liquor	Acetone	0	1·7	7·7	8·6	0·5	0	7·0	11·3	18·5	21·2	0·9
	Isopropanol	0	4·1	6·6	5·4	1·3	0	3·7	3·5	4·3	3·1	1·4
	Acetoacetic Acid	0	0·1	2·8	4·5	1·3	0	1·2	1·7	1·2	3·5	0·3
	β-hydroxybutyric Acid	2·3	2·0	4·4	21·3	0·3	2·5	4·8	1·3	5·2	2·9	7·4
Blood	Acetone	0	5·2	10·8	16·2	2·3	0	7·0	11·3	21·2	23·4	0·9
	Isopropanol	0	0·3	1·0	0·8	0	0	0·7	0·8	1·6	1·3	0
	Acetoacetic Acid	0	0·6	1·3	1·6	2·2	0	4·6	2·9	3·5	3·7	0·3
	β-hydroxybutyric Acid	4·5	14·5	11·1	3·7	2·3	7·8	4·4	3·8	1·4	7·1	4·5
Milk	Acetone	0	2·5	12·2	15·5	4·6						
	Isopropanol	0	0	1·3	1·8	0						
	Acetoacetic Acid	0	1·2	5·4	7·2	1·9						
	β-hydroxybutyric Acid	2·1	11·8	6·0	0·1	1·7						
Urine	Acetone	0	4·2		18·0							
	Isopropanol	0	0		1·5							
	Acetoacetic Acid	1·0	1·1		1·1							
	β-hydroxybutyric Acid	20·9	16·8		22·1							

levels of acetoacetic acid were found in the blood and of acetone in the rumen, while the levels of isopropanol in the rumen were lower.

Repeated administration of isopropanol into the rumen. In the first experiment with cows large doses of isopropanol were administered, viz. 400 ml. one day and 250 ml. the next day. The values for total ketone bodies were too high to estimate with any accuracy, but there was no clinical upset. The animals were left for four days and the experiment was repeated using smaller doses, viz, 100 ml. and 150 ml. isopropanol with the results shown in Table 6. It will be noted that appreciable quantities of ketone bodies were still found in the blood and rumen at the start of the second experiment. During the period of administration there was a steady rise in isopropanol and acetone levels in the rumen liquor followed by a gradual fall when the injections ceased. Approximate parity of acetone and isopropanol was maintained in rumen contents until the third day when isopropanol became predominant. Blood acetone levels were at all times higher than those of isopropanol. The level of acetoacetic acid and β-hydroxybutyric acid in both blood and rumen contents remained low and fairly steady throughout the experiment, i.e. with the method of sampling used (once daily before the first dose) the rapid conversion of ruminal isopropanol into β-hydroxybutyric acid, noted in the single dose experiments, was not observed. The changes in ketone levels in the milk followed those in the blood except that the build-up of β-hydroxybutyric acid in milk was considerably greater than in blood. Though the values for the sheep were all lower than for the cow the general trend of the changes was the same.

Repeated injection of ethyl acetoacetate intravenously in the cow. Owing to the toxity of ethyl acetoacetate it was not thought advisable to inject more than 30 ml. at a time, so this dose was given four times daily, viz. at 9.30 a.m., 11.45 a.m., 2.0 p.m., and 4.45 p.m. On the first two days of the experiment samples taken in the morning, before the first injection of the day, showed little or no change from the normal. On the third day, therefore, samples were taken just before the third and fourth injections of that day. On both occasions relatively small amounts of acetone, acetoacetic acid and isopropanol were found in the blood and rumen liquor (Table 7). It is interesting to note that the levels of isopropanol were higher in the rumen liquor than in the blood; β-hydroxybutyric acid was the most prominent ketone body in both the blood and the rumen. Traces of acetone bodies were found in the milk while β-hydroxybutyric acid was the main excretory product in the urine. Although acetone exceeded acetoacetic acid in the blood and rumen liquor the reverse was true in the urine. In general the results indicated a rapid metabolism of the acetoacetate injected without any marked formation of other ketone bodies.

TABLE 6

KETONE BODY LEVELS FOLLOWING THE ADMINISTRATION OF REPEATED DOSES OF ISOPROPANOL INTO THE RUMEN

Expressed as mg. acetone/100 ml.

		Days of experiment								
		0	*1*	*2*	*3*	*4*	*5*	*7*	*8*	*9*
	Isopropanol injected	*4 × 25 ml.*	*4 × 30 ml.*	*4 × 35 ml.*	*3 × 50 ml.*	*4 × 75 ml.*				
Cows										
Rumen Liquor	Acetone	5·4	9·0	12·6	14·0	20·3	33·3	5·2	5·9	1·4
	Isopropanol	5·4	10·3	14·5	43·0	110·0	52·5	11·0	10·5	1·4
	Acetoacetic Acid	0·4	0·7	0·6	0·8	1·3	4·2	3·8	0·7	0·5
	β-hydroxybutyric Acid	8·0	3·5	1·1	2·1	8·4	4·2	1·6	1·4	3·2
Blood	Acetone	7·9	14·4	22·5	27·5	26·1	45·5	12·6	1·8	1·4
	Isopropanol	0·2	0·8	2·7	4·5	4·1	14·0	0·9	0·0	0·0
	Acetoacetic Acid	0·8	4·1	4·0	5·2	4·7	2·5	2·8	1·0	0·5
	β-hydroxybutyric Acid	13·9	6·9	9·2	12·0	13·7	0·3	8·8	12·9	12·1
Milk	Acetone	10·8	14·0	27·1	29·3	27·0	47·4	13·3	2·0	0·0
	Isopropanol	0·0	0·6	1·9	3·5	8·2	8·5	1·1	0·0	0·0
	Acetoacetic Acid	0·3	2·4	0·2	1·5	9·9	12·2	0·6	2·6	4·6
	β-hydroxybutyric Acid	9·7	12·8	20·7	30·1	45·3	21·8	22·8	8·7	3·9
Sheep	*Isopropanol injected*	*4 × 5 ml.*	*4 × 5 ml.*	*4 × 5 ml.*	*4 × 10 ml.*					
Rumen Liquor	Acetone	0·0	8·3	9·6	4·9	19·4	0·0			
	Isopropanol	0·0	3·0	3·5	3·4	4·1	0·0			
	Acetoacetic Acid	0·0	0·2	0·5	0·5	2·8	0·0			
	β-hydroxybutyric Acid	10·3	6·5	4·4	12·0	8·6	1·9			
Blood	Acetone	2·7	8·3	8·4	7·0	17·1	0·0			
	Isopropanol	0·0	0·3	1·5	0·6	1·0	0·0			
	Acetoacetic Acid	0·3	0·3	2·0	0·7	3·8	0·0			
	β-hydroxybutyric Acid	13·5	19·3	17·8	19·0	2·5	10·5			

TABLE 7

KETONE BODY LEVELS FOLLOWING REPEATED INTRAVENOUS INJECTIONS OF ETHYL ACETOACETATE IN THE COW

Expressed as mg. acetone/100 ml.

	Ketone body	*0 day 9.0 a.m.*	*1st day 9.0 a.m.*	*2nd day 9.0 a.m.*	*3rd day*	
					1.50 p.m.	*4.40 p.m.*
Rumen Liquor	Acetone	0	0		0·5	0·5
	Isopropanol	0	0		0·6	0·4
	Acetoacetic Acid	0	0		0·3	0·3
	β-hydroxybutyric Acid	2·2	2·9		7·8	2·2
Blood	Acetone	0	0	0	0·9	0·9
	Isopropanol	0	0	0	0	0·1
	Acetoacetic Acid	0	0	0	0·3	0·5
	β-hydroxybutyric Acid	4·1	15·4	9·4	12·7	7·6
Milk	Acetone	0	0		0	1·4
	Isopropanol	0	0		0	0·6
	Acetoacetic Acid	0	0		1·9	0·5
	β-hydroxybutyric Acid	6·7	4·8		14·9	5·7
Urine	Acetone	0	0		1·6	2·1
	Isopropanol	0	0		0	0·8
	Acetoacetic Acid	0	0		3·1	3·1
	β-hydroxybutyric Acid	6·4	6·4		13·8	6·8

DISCUSSION

The effects of administering the various ketone bodies to cows and sheep were the same qualitatively though not quantitatively. This may be associated with the fact that the cows used were all in milk while the sheep were in a non-pregnant non-lactating state. Moreover, the dosage rate per unit of body weight was less with sheep than with cows. The cows used were not milked out at each sampling so the results obtained for the milk were cumulative over twelve hours. As in all cases the ketone levels in the milk fell within about six hours after administration, either the acetone bodies were utilised by the mammary gland or diffusion back into the blood took place. Shaw (1942) has shown that the mammary gland can utilise β-hydroxybutyric acid, but not acetoacetic acid, for energy purposes. As acetoacetic acid was found in higher concentrations in the milk than in the blood in these experiments, this would seem to support the view of non-utilisation of this substance by the gland, accompanied possibly by an inability to diffuse back into the blood. From the figures obtained it seems that the total amount of ketone bodies excreted in the milk and urine in no way accounted for all the specific ketone body administered. A fairly large proportion of the more volatile ketones appeared to be excreted through the lungs while the remainder of the ketone bodies, in one form or another, were probably metabolised by the body to other compounds. Holmes (1950) and Schultz and Smith (1951) showed that there was a rise in blood ketones after the oral administration of acetone. Holmes found that acetone was rapidly excreted from the mammary gland, but found no detectable amounts in the urine even after a dose of 500 ml. acetone. By contrast, in the above experiments where much smaller amounts of acetone were given, a proportion was excreted by the mammary gland, but within an hour of administration a high percentage of acetone was excreted in the urine suggesting that this substance has a low renal-threshold value.

Using radioactive isotopes, Plaut and Lardy (1950) demonstrated that acetone could be incorporated into acetoacetic acid in rat liver slices. It appears reasonable to suppose that a similar reaction can take place in ruminant tissues and in the rumen as after administration of acetone to ruminants, both intravenously and into the rumen, acetoacetic acid was found. Decarboxylation of acetoacetic acid with the formation of acetone also occurs; an enzyme has been demonstrated in *Clostridium acetobutylicum* which catalyses the decarboxylation of acetoacetic acid (Johnson, Peterson and Fred, 1933). A similar catalyst has been claimed to occur in dogs' blood (Rossi, 1938), and it may well be that the same reaction occurs in ruminant tissues and in the rumen.

An interesting feature of the trials was the slight response to the administration of β-hydroxybutyric acid and of methyl acetoa-

cetate. For example, with administration of a single dose of these two compounds into the rumen, although high ruminal concentrations were maintained for some hours the effects on the blood levels of the substance administered were very slight and there was no evidence of any extensive conversion to other ketone bodies. A similar trend was evident with the intravenous injections; here there was apparent rapid utilisation without any marked production of other ketone bodies. This was particularly true of β-hydroxybutyric acid where, after administration either into the rumen or the blood, no other ketone bodies were detected. With acetoacetate, Schultz and Smith (1951) found with the sodium salt given orally a gradual increase in blood ketones while the ethyl ester, given either orally or subcutaneously, caused some increase in total blood ketones. Clark and Malan (1956) showed that the rise in blood acetoacetic acid, after the intravenous injection of acetoacetate, was only transitory. They also demonstrated the conversion of acetoacetic acid to β-hydroxybutyric acid in vivo and in vitro in sheep's blood. The above experiments suggest that the conversion of acetoacetic acid to β-hydroxybutyric acid can take place in the rumen as well as in ruminant tissues and on the whole appears to take place with greater ease than conversion to acetone. There is ample evidence to show that both β-hydroxybutyric acid and acetoacetic acid may be catabolised to CO_2 and H_2O by isolated slices of kidney muscle, heart, brain, testis, etc, in vitro; similar evidence is available for perfused whole organs (Soskin and Levine, 1952). The disposal capacity of the body for acetoacetic acid and β-hydroxybutyric acid is believed to be considerable and, certainly, in the present trials appeared adequate to deal effectively with the amounts of β-hydroxybutyrate and acetoacetate administered.

Several workers have mentioned the toxicity of acetoacetate, e.g. Huntly (1916), Allen and Wishart (1923), Schnieder and Droller (1938) and Chari and Wertheimer (1954). Our experiments showed that large doses of acetoacetate produce a comatose condition and cause considerable respiratory distress. The levels of acetoacetic acid in the blood appear to be kept low, partly by conversion to acetone and β-hydroxybutyric acid, neither of which appeared to be toxic at the levels reached. Acetoacetic acid is also rapidly excreted through the lungs and urinary tract. From the amount of acetoacetic acid excreted in the cow's urine after an intravenous injection of acetoacetate, it appears that the renal-threshold for this substance is very low in the cow; this would be an additional protective measure. If large quantities of acetoacetic acid appeared in the blood, as after injection, the normal detoxification reactions of decarboxylation to acetone and reduction to β-hydroxybutyric acid would not be able to act with sufficient speed to prevent the toxic action without the excretion of a large proportion of the acetoacetic acid through the urine and lungs taking place at the same time.

A survey of the literature shows there is some difference of

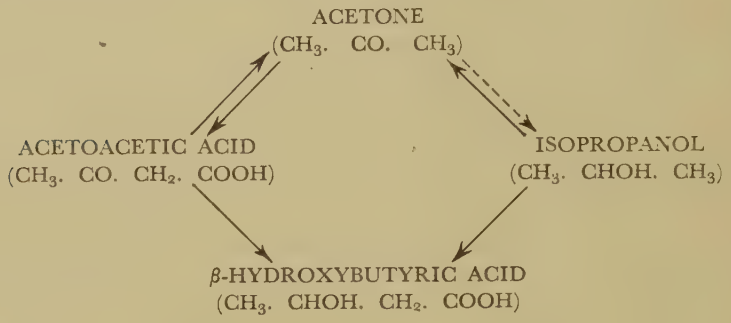
opinion regarding the metabolic path of β-hydroxybutyric acid in the ruminant. Barnes, MacKay, Moe and Visschers (1938) and Schultz and Smith (1951) found β-hydroxybutyric acid was metabolised without the production of acetone or acetoacetic acid. Knodt and Petersen (1946) and Peeters, Coussens and Sierens (1953) found that during incubation and perfusion of the mammary gland both acetone and acetoacetic acid were produced from β-hydroxybutyric acid. Clark and Malan (1956) noted a slight rise in blood acetoacetic acid and acetone after an intravenous injection of β-hydroxybutyric acid. In the above experiments it was found that β-hydroxybutyric acid, given by intravenous injection or orally, disappeared without the production of any of the other ketone bodies; further, it was the only ketone body present before and after the experiment. As, on the whole, the administration of the other ketone bodies caused a rise in β-hydroxybutyric acid levels, it would appear that this ketone is the normal end point in ketone body interconversions in the ruminant. As this ketone is present in the normal animal it is presumably harmless, and so conversion to β-hydroxybutyric acid could be regarded as a protective mechanism.

Since it was first recorded by Robertson, Thin and Stirling (1950) that isopropanol occurs in ruminant ketosis, Adler, Roberts and Dye (1955) reported that 100 to 300 ml. of isopropanol, given by intravenous injection to a cow, caused marked toxic symptoms. From the present series of experiments it appears that 250 ml. isopropanol per os, or 125 ml. intravenously, can be metabolised in the rumen and tissues of the ruminant with the production of all the other ketone bodies without the production of toxic symptoms. It appears that isopropanol can be formed in the rumen from acetone probably by bacterial action though, perhaps, it may be produced like other ketone bodies, by the rumen epithelium (Pennington, 1951). In most of these experiments isopropanol appeared in the rumen before it appeared in the blood and was found in highest concentrations in the rumen. It is also evident from our findings that the interconversion of acetone and isopropanol, as well as the conversion of isopropanol to β-hydroxybutyric acid, takes place very easily in the rumen.

On the basis of the above results we would suggest the following as the likely cycle of events in inter-ketone body conversion in the ruminant, the solid lines indicating activities in blood, tissues and rumen while the reaction shown by the dotted line may be limited to rumen only.

If the current view of the causation of ketosis in diabetes were applicable to ruminants one would anticipate that the most suitable way of simulating ruminant ketosis would be by the repeated injection of intravenous acetoacetate. It is of some interest, therefore, to compare the blood, rumen, milk and urine ketone levels obtained by repeated acetoacetate injection with those occurring

in starvation (Robertson and Thin, 1953) and in clinical ketosis (Thin and Robertson, 1953).



As mentioned earlier successive injections into a cow of 30 ml. ethyl acetoacetate produced only transient ketonaemia and there was little cumulative effect with four daily doses. Comparing the injection with starvation there is a close similarity for blood, rumen liquor and milk. The levels of acetoacetic acid and of β-hydroxybutyric acid in the urine, however, differ quite markedly being much higher with starvation. This suggests that the output of acetoacetate in starvation is much greater than has been achieved by the four intravenous injections which amounted to a daily total of 120 ml. of ethyl acetoacetate. The higher levels of acetoacetate and β-hydroxybutyric acid in urine in starvation, however, do not suggest that there is any intrinsic difference in metabolic pattern during starvation from that to be expected when high levels of blood acetoacetate are maintained. Successive injections every three hours can only approximate to a steady high output of acetoacetate and this difference is probably reflected in the urine concentrations.

In comparison with the results from clinical cases even those showing the lower levels of total ketone bodies—Group C, Thin and Robertson (1953)—were on average some 2 to 3 times as great in total ketones in blood, rumen liquor and milk as those obtained by injection, while the urine concentrations were some seven times as great. As with starvation the relative proportions of the various ketone bodies in mild ketosis and following acetoacetate injection do not differ markedly and such differences as have been observed may well be connected with the effect that high levels of total ketone bodies appear to have on the proportions of acetone, isopropanol and β-hydroxybutyrate.

The results with acetone and isopropanol administration contrast with those for β-hydroxybutyrate and acetoacetate. Here, there was an appreciable blood response to the substance administered into the rumen and also evidence of conversion into other ketone bodies. Isopropanol, as judged from the results with a single ruminal dose, appeared capable of yielding within a few

minutes large amounts of β-hydroxybutyric acid, the level of which returned to normal after two hours. This effect was not evident in the repeated doses trial, probably because of the 12-hour interval between the last daily dose and sampling. Interconversion of acetone and isopropanol is also indicated by the results; differences in oxygen tension or differences in absorption rate may account for the marked preponderance of acetone in blood as compared with the approximate equivalence of acetone and isopropanol in rumen contents which occurred with both the repeat doses of intravenous acetone and of intra-ruminal isopropanol. Both substances were found capable of giving high levels of total blood ketones without ill effect on the subject.

After the repeated administration of acetone intravenously, or isopropanol into the rumen, the levels of both acetone and isopropanol in the blood were higher than those found in starvation ketosis or clinical ketosis, although in the case of isopropanol in the blood after acetone administration the difference was not marked. In both administration experiments the levels of both acetoacetic and β-hydroxybutyric acids in the blood were less than those in starvation ketosis or clinical ketosis. The values for total acetone bodies found in the rumen liquor were higher after these two repeated administration experiments than during starvation ketosis or clinical ketosis for corresponding values of total blood ketones. There was also a much higher proportion of both acetone and isopropanol in the rumen liquor and in the milk after both administrations. After the administration of acetone intravenously the urine contained more acetone and isopropanol, but much less acetoacetic acid than in starvation or clinical ketosis.

From these results it would appear improbable that the range of ketones occurring in clinical cases can be derived from either isopropanol or acetone as the primary substrate. Acetoacetate would appear to be the more likely but in that case the amounts produced in the disease condition seem to be considerably greater than could be accounted for by the anorexia observed. Moreover, the relatively large amounts of isopropanol in rumen liquor and urine in clinical cases as compared with starvation or acetoacetate injection suggest that other factors such as abnormal rumenal conditions may be involved in clinical ketosis.

CONCLUSIONS

The administration of acetone, acetoacetate and isopropanol separately to the ruminant, by mouth or intravenous injection, was found to produce a transient form of ketonaemia.

A high degree of interchange was found to occur between these ketone bodies in the blood, tissues and rumen. The latter organ appeared to be the main, if not the only, site of formation of isopropanol.

The administration of β-hydroxybutyrate produced no ketones and this fraction appeared to be the end-point of inter-ketone body conversion.

A cycle of events is suggested for the metabolism of the ketone bodies in the blood and tissues and in the rumen.

The chemical form of ketonaemia obtained in the ruminant subjected to frequent administrations of a ketone body is compared with clinical ruminant ketosis and that produced by starvation in the ruminant.

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THE COMPLEMENT-FIXING CAPACITY OF AQUEOUS EXTRACTS OF MYCOBACTERIA

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INTRODUCTION

As an aid to diagnosis of human and bovine tuberculosis and particularly of Johne's disease in cattle and sheep, complement fixation tests have been widely applied, the antigens used being whole bacilli (Hole, 1956), and methanolic (Négre and Boquet, 1921) or aqueous extracts (Wadsworth, Maltaner and Maltaner, 1925). Aqueous extracts are easy to prepare and have proved useful in studies of mycobacterial infections at Weybridge (Report, 1955). These extracts were originally described by Wadsworth, *et al.* (1925) who showed in a detailed study that the activity of human and bovine strains probably resided in the phosphatide fraction of the bacillus. Later work from the same laboratory with human type bacilli suggested a similarity of active principle in both aqueous and methanolic extracts (Pangborn, 1956).

With the object of obtaining a reproducible antigen containing known components of mycobacteria, we have attempted to correlate the serological activity of aqueous extracts with their chemical constitution. These extracts, in addition to being easily prepared, are less anticomplementary than certain methanolic extracts. The present paper describes a quantitative study of the chemical composition of Maltaner-Wadsworth extracts from a range of mycobacterial types, the specificity of the extracts, and the correlation between chemical analytical results and serological activity.

METHODS

Strains of Mycobacteria. Human strains, PN, DT and C; bovine strains, AN5, Otto, CB17; avian strains, D4ER, V64; Johne strains, 3 and 5 (Path.), RLE, Teps; saprophytes, *M. fortuitum, M. smegmatis.*

Media. All strains were grown on synthetic medium (Dorset, 1934; Watson, 1935) with the exception of two human strains, which were cultured in addition on a conventional glycerol-beef-broth medium.

Preparation of antigens. Bacilli were harvested at 4 to 5 weeks of growth, suspended in 5 per cent phenol at 4°C for 24 hours, thoroughly washed and freeze-dried. The organisms were shaken with two changes of acetone (100 ml. acetone to 1 g. bacteria) at room temperature, filtered and air-dried. The dried bacteria were boiled for 1 hour with water under reflux with stirring and then filtered off on a Whatman No. 14 paper. An aliquot of the filtrate was centrifuged at 12,000 r.p.m. in an M.S.E. angle head centrifuge for 30 minutes at room temperature. Tests and analyses were carried out on supernates and deposits, all results being referred to a dry-weight basis. Antigen preparations were

dried from the frozen state and stored at 4°C prior to making chemical analyses. When required, the antigens were reconstituted in water at a strength of about 1 mg./ml. (in practice 15 to 25 mg. freeze-dried powder were dissolved in 25 ml. water). Serological tests were carried out before freeze-drying.

Chemical analyses. Dry weight; 2 ml. of antigen solution was dried in an electric oven at 120°C and the residue cooled to constant weight in a desiccator over calcium chloride. Total nitrogen was determined by the micro-Kjeldahl method, and distillations carried out in a Markham apparatus 5 ml. of solution being used. Total phosphorus: the method of Allen (1940) was applied to 1 ml. of solution. Protein nitrogen was determined by the colorimetric method of Lowry, Rosenbrough, Farr and Randall (1951) using 0·6 ml. Hexoses: the anthrone colorimetric method of Trevelyan and Harrison (1952) was used for determining hexoses, on 1 ml. of a 2N acid hydrolysate of the antigen solution, equivalent to 0·5 ml. original solution. Pentoses: a quantitative version of the Bial orcinol reaction was used to estimate pentoses with a modification of the reagent of Mejbaum (1939), described by Albaum and Umbreit (1947). The amount used was 0·25 ml. Total carbohydrate was determined by addition of hexose and pentose and ignoring the small errors arising from interaction between hexoses and the pentose reagent. Ultraviolet absorption spectra were determined in a Hilger and Watts Uvispek ultra-violet absorptiometer.

Complement-fixation tests. Antigens were titrated using excess of antiserum, 50 per cent end-points being determined colorimetrically. Each antigen was tested against three standard sera prepared in cattle against bovine, avian and Johne type mycobacteria. The bovine antiserum was a pooled sample obtained from cattle experimentally infected with bovine tuberculosis, the avian produced by inoculation of live avian type organisms intramuscularly in oil, and the *M. johnei* antiserum by repeated intravenous inoculation of live *M. johnei*. Antigens and sera were heated at 60°C for half an hour before testing.

Doubling dilutions of antigen solution in the range 1 in 16 to 1 in 2,048 were prepared in barbiturate buffer (Brooksby, 1952) and 0.25 ml. of each of these dilutions transferred to 8 × 1 cm. matched optical density tubes (Evans Electroselenium Limited). Antiserum (0·25 ml.) diluted 1 in 10 in buffer was added to all tubes. Burroughs Wellcome preserved guinea-pig complement (0·25 ml.) containing 2 × 50 per cent units was added to all tubes. The contents were mixed by shaking and incubated at 37°C for 1 hour. The haemolytic system (0·25 ml.) was added to all tubes and dispersed by shaking. The sheep cells were sensitized by incubating a 6 per cent suspension with equal volumes of 10 MHD haemolytic serum (B.W. glycerinated) for 30 minutes at 37°C. The tubes were incubated at 37°C, shaking at intervals to suspend unhaemolyzed cells, until control tubes showed complete haemolysis (approximately 40 mins.). The buffer solution (2·0 ml.) was added to all tubes with an automatic syringe, the jet effecting complete mixing. This gave an optimal 3·0 ml. for colorimetric reading. All tubes were centrifuged at 2,000 r.p.m. for 3 minutes to deposit suspended unhaemolysed cells. Readings were made on an E.E.L. colorimeter fitted with a test-tube adaptor, the buffer control being used as a blank for zero setting. The reading for 100 per cent haemolysis was determined by averaging

the reading of tubes showing complete haemolysis. The percentage haemolysis in any individual tube was then tube reading/100 per cent haemolysis reading × 100. The volume of antigen solution at the 50 per cent end-point was determined by graphical interpolation.

The controls for each test were (1) 0·25 ml. 1/10 serum, 0·25 ml. buffer, 0·25 ml. complement, 0·25 ml. haemolytic system; (2) 0·25 ml. 1/10 antigen, 0·25 ml. buffer, 0·25 ml. complement, 0·25 ml. haemolytic system; (3) 0·75 ml. buffer, 0·25 ml. haemolytic system; (4) 0·5 ml. buffer, 0·25 ml. complement dilution, 0·25 ml. haemolytic system.

The activity of each antigen was expressed as actual weight dry antigen in micrograms at the 50 per cent end-point. The smaller the weight the greater is the activity.

RESULTS

Serological Activity

Table 1 summarizes the results of tests carried out on the various antigen preparations. The first obvious point is that the activity of an antigen prepared from the same strain on different occasions varies widely. Secondly, the activity against bovine antiserum of all antigens is generally higher than that against Johne or avian antiserum, but this is only obvious when a number of preparations are examined because of the wide activity variation of individual preparations. Bovine antigens show the highest activity against homologous but not heterologous antisera, Johne antigens against homologous and heterologous, while avian shows highest activity against bovine only. Human and saprophytic antigens resemble the Johne extract in having high activity against all three sera.

Centrifugation of the aqueous extracts led to a slight but non-significant increase in activity, the deposited material suspended in the same liquid volume from which it was sedimented having little or no activity.

Chemical Composition and Serological Activity

Table 2 shows the material extracted by successive treatments with acetone and boiling water. The amounts extracted from the various types vary widely and are not characteristic of type. The acetone soluble lipid is serologically inactive.

All antigen preparations were analyzed for nitrogen, phosphorus, protein nitrogen, and carbohydrate but, in addition, more extensive analyses and fractionations were carried out on freeze-dried powders obtained from the aqueous extracts, both before and after high-speed centrifugation. Table 3 shows the proportion of the dry weight sedimented and Table 4 the composition of the aqueous extracts of organisms grown on synthetic media. Within the limits of analytical error, no alteration in composition was obvious after centrifugation, largely because of the small proportion of material sedimented, which was of the same order as the analytical reproducibility. When the human strains C and PN were grown on glycerol-

TABLE 1

THE SEROLOGICAL ACTIVITIES OF THE AQUEOUS EXTRACTS† OF MYCOBACTERIA

Strain from which antigen was prepared (*No. of preparations*)	*Complement fixing activity* (*μg. dry wt. at end-point*) *Average figures and ranges*		
	Anti-bovine serum	*Anti-Johne serum*	*Anti-avian serum*
Bovine			
Otto (4)	5·9* (0·6 – 12·4)	27·4 (16·8 – 37·5)	29·1 (19·0 – 37·5)
AN_5 (6)	6·7 (3·1) – 10·8)	23·9 (10·8 – 30·0	24·1 (12·0 – 30·0)
CB_{17} (4)	3·0* (1·0 – 5·0)	25·0 (22·5 – 27·5)	18·6 (16·2 – 21·0)
Johne			
Teps (6)	1·4* (0·1 – 4·7)	1·5* (0·9 – 1·9)	2·2* (0·3 – 6·4)
RLE (4)	0·8* (0·2 – 2·3)	1·8* (1·3 – 2·9)	1·3* (0·4 – 2·2)
3 & 5 Path. (6)	3·2* (1·7 – 5·5)	18·0 (12·4 – 23·2)	24·7 (22·5 – 30·0)
Avian			
D4ER (4)	7·4 (6·0 – 9·6)	28·8 (30·0 – 35·0)	28·8 (30·0 – 35·0)
V64 (4)	2·1* (0·4 – 3·4)	8·2 (4·6 – 15·0)	12·7 (10·9 – 15·0)
Human			
C (8)	0·9* (0·3 – 1·1)	6·6 (1·3 – 18·5)	2·5* (2·1 – 3·3)
Saprophytic			
Smegmatis (2)	1·4* (1·0 – 1·7)	7·7 (5·5 – 9·8)	1·3* (1·2 – 1·5)
Fortuitum (4)	0·7* (0·2 – 1·2)	0·3 (0·2 – 0·4)	0·3* (0·2 – 0·5)

† Simple aqueous extracts and centrifuged extracts.
* These groups of results were used in statistical treatments.

beef-broth, 40 to 50 per cent of the dry weight of simple aqueous extracts was deposited on centrifugation and a difference in composition of the supernatant fluid was detected. Total nitrogen and protein nitrogen were lowered and total phosphorus and total carbohydrate raised, suggesting that protein-rich, phosphorus and carbohydrate-poor material was sedimented. The sediment in these cases showed some degree of serological activity. The variation in

composition was so wide that no obvious relationship between type of powder and analytical results emerged.

Antigen solutions showed absorption of ultra-violet light over the wavelength range 230 mμ to 320 mμ. Aqueous extracts and centrifugates usually showed a well-defined absorption maximum at about 255 mμ and a minimum at about 240 mμ. Little absorption occurred beyond 320 mμ. The absorption curves showed no features characteristic of individual antigen preparations.

TABLE 2

SUCCESSIVE EXTRACTIONS WITH ACETONE AND BOILNG WATER

Strain from which antigen prepared	*Medium*	*Approx. % dry wt. of freeze-dried orgns. extracted with acetone at R°*	*Approx. % dry wt. of acetone extracted orgns. extracted with water at 100°*
Human			
C ..	Synth.	—	16·1
		24	19·4
		26	17·0
		30	21·2
C ..	Beef	20	27·1
PN ..	Synth.	—	10·4
		20	15·2
PN ..	Beef	20	18·7
Bovine			
AN_5 ..	Synth.	20	11·1
		12	7·7
		16	10·8
Otto ..		20	9·6
		16	12·6
CB17 ..		10	9·7
		8	8·8
Avian			
D4ER ..	Synth.	10	11·9
		9	11·0
V64 ..	Synth.	24	6·6
		12	5·6
Johne			
3 & 5 Path. ..	Synth.	30	8·4
		32	9·5
		40	9·0
RLE ..	Synth.	20	6·9
		20	9·4
Teps ..	Synth.	20	4·9
		10	8·3
		13	5·8
Saprophytic			
Fortuitum ..	Synth.	10	11·4
		8	13·0
		13	11·0
Smegmatis ..	Synth.	13	5·2

TABLE 3

SEDIMENTATION OF EXTRACTS AT 12,000 R.P.M. FOR 30 MINS.

Strain from which antigenic extract prepared (No. of prepns.)	*Medium*	*% dry wt. sedimented at 12,000 r.pm./30 m. Average and range*
Human		
C (4)	Synth.	6·1 (3·4 – 10·7)
C (1)	Beef	53·3
PN (2)	Synth.	4·4 (2·8 – 5·9)
FN (1)	Beef	41·4
Bovine		
AN_5 (3)	Synth.	3·8 (1·6 – 6·2)
Otto (2)	,,	2·3 (1·9 – 2·7)
CB17 (2)	,,	2·2 (2·0 – 2·4)
Avian		
D_4ER (2)	,,	2·7 (1·4 – 3·9)
V64 (2)	,,	4·8 (3·9 – 5·7)
Johne		
3 & 5 Path. (3)	,,	5·7 (3·2 – 8·7)
RLE (2)	,,	3·7 (3·6 – 3·7)
Teps (3)	,,	2·4 (2·4 – 2·5)
Saprophytic		
Fortuitum (3)	,,	5·0 (2·3 – 8·3)
Smegmatis (1)	,,	9·6

The Nature of the Active Fraction

In keeping with the lack of characteristic chemical analyses of the antigens, no correlation could be found between analyses for specific substances such as protein or carbohydrate and complement-fixing activity. If, however, the difference between total analyzed material and total weight of material was determined, a significant correlation between this difference and complement-fixing activity was demonstrable. This fraction has been called the unanalysed fraction or "U.A.F." In determining this correlation only the more active preparations were used (asterisked in Table 1), that is, those showing titres of greater than 1 in 50 with solutions containing

TABLE 4

CHEMICAL ANALYSES OF FREEZE-DRIED ANTIGEN POWDERS

Obtained from simple aqueous extracts of 12 strains of Mycobacteria before and after centrifuging at 12,000 r.p.m. for 30 mins

Type	Strain from which antigen prepared	Simple aqueous extracts					Centrifuged extracts					No. of prepns. made
		% N	% P	% Protein–N	% Pentoses*	% Hexoses	% N	% P	% Protein-N	% Pentoses*	% Hexoses	
Human	C	3·77	5·03	1·49	6·40	27·32	4·42	5·28	1·57	6·84	29·55	4
		3·44–4·29	4·28–5·47	1·40–1·70	5·10–7·21	24·13–30·30	4·00–5·07	4·98–5·81	1·36–1·91	6·02–7·31	26·60–34·73	
	C (Beef)†	5·98	2·16	3·58	5·90	13·30	4·48	2·80	2·41	6·85	19·90	1
	PN	4·28	4·22	2·03	6·40	13·04	4·53	3·80	1·91	6·38	14·11	3
		3·11–4·90	4·23–4·40	0·93–3·05	4·25–7·60	7·83–16·58	2·83–6·66	3·09–4·50	1·09–2·77	5·13–7·32	10·68–18·45	
	PN (Beef)	6·17	1·91	3·80	5·67	13·90	4·24	2·75	2·44	6·11	18·08	1
Bovine	AN_5	2·89	2·18	1·47	3·68	14·50	3·05	2·37	1·48	3·88	15·22	3
		2·47–3·68	2·05–2·27	1·25–1·85	3·53–3·80	12·64–16·10	2·81–3·13	2·25–2·53	1·28–1·84	3·59–4·14	13·16–17·66	
	Otto	2·90	2·55	1·40	3·07	11·33	2·91	2·48	1·45	3·12	11·72	2
		2·67–3·12	2·45–2·65	1·16–1·63	2·86–3·28	9·33–13·32	2·81–3·00	2·42–2·53	1·20–1·70	2·88–3·35	11·22–12·22	
	CB17	2·67	2·89	1·21	4·72	8·44	2·47	2·81	1·58	4·49	8·18	2
		2·55–2·78	2·60–3·18	1·19–1·23	4·48–4·95	5·32–11·56	2·39–2·54	2·54–3·07	1·20–1·96	4·35–4·63	5·45–10·90	
Avian	D4ER	1·59	0·96	0·92	7·65	18·59	1·72	0·98	1·00	8·37	19·83	2
		1·10–2·08	0·86–1·05	0·69–1·14	7·44–7·85	16·28–20·90	1·43–2·00	0·97–0·98	0·91–1·08	7·56–9·17	19·48–20·18	
	V64	1·85	1·73	0·81	7·13	21·75	1·71	1·81	0·74	7·87	22·62	2
		1·64–2·06	1·66–1·80	0·75–0·86	6·10–8·15	17·97–24·52	1·35–2·06	1·65–1·96	0·70–0·77	7·06–8·67	19·00–26·23	
Johne	3 & 5	2·84	3·36	1·74	11·38	25·62	3·02	3·38	1·69	11·02	23·87	3
		2·00–3·86	3·06–3·78	1·45–2·06	9·63–13·63	23·30–28·85	2·46 3·47	2·87 4·02	1·51–1·84	9·79–12·42	23·38 24·50	
	RLE	5·20	4·62	2·48	5·11	7·88	5·10	3·93	2·32	4·53	6·87	2
		4·60–5·80	4·11–5·13	1·93–3·02	4·90–5·32	6·39 9·36	4·94–5·26	3·89 3·97	1·97 2·67	4·36–4·70	5·86–7·88	
	Teps	5·70	4·94	2·67	5·75	9·39	6·17	5·26	2·54	5·59	8·78	3
		4·48–7·73	4·19–6·00	2·05–3·75	4·88–7·12	6·04–14·78	4·82–8·52	4·24–6·18	2·01–3·56	4·74–7·13	5·45–13·71	
Saprophytic	Fortuitum Smegmatis	2·03	3·42	0·93	3·85	7·05	2·49	3·44	0·99	3·69	6·41	3
		1·54–2·28	3·31–3·58	0·89 1·06	3·53–4·41	5·24 8·49	2·45 2·58	3·17–3·90	0·92–1·03	3·10–4·31	5·08–7·52	
		3·16	3·67	1·57	4·59	13·02	3·57	4·04	1·61	4·99	13·62	1

* Pentose figures not corrected for interference from hexoses. †When not specified, the organisms were grown on synthetic media.

The figures give mean results and ranges.

about 1 mg/ml. Table 5 shows that highly significant correlations existed between the UAF of antigens and their activity against bovine and avian sera. The same trend was shown with anti-Johne sera, but the correlation was of low order of significance ($P = 0.2$). There was no evidence of type specificity, since the activity of antigens against bovine and avian sera showed the positive correlation with UAF percentages regardless of the source of the antigen.

TABLE 5

THE RELATIONSHIP BETWEEN COMPLEMENT-FIXING ACTIVITY AND UAF

Relationship between C.F. activity & UAF	*No. of samples*	*Correlation coefficient r*	*t (calc.)*	*Value of t (n–2) at P.05*	*P.01*	*Significance*
Against anti-Avian serum*	21	–0·81	–6·02	2·09	2·86	P ·01
Against anti-Bovine serum*	38	–0·46	–3·12	2·03	2·71	P ·01
Against anti-Johne serum*	20	–0·31	–1·38	2·10	2·88	Not sig.

* Antigens giving a titre of more than 1/50 with an approx. 1 mg/ml. solution of freeze-dried antigen powder.

UAF = unanalysed fraction.

TABLE 6

COMPARISON BETWEEN UAF AND THE FRACTIONS OF SOME FREEZE-DRIED ANTIGENS SOLUBLE IN ORGANIC SOLVENTS

Antigen No.	*Strain from which prepared*	*Et_2O-EtOH (1:1) soluble material % dry wt.*	*MeOH soluble material % dry wt.*	*UAF % dry wt.*
44	RLE	29·4	—	71·1
49	D4ER	52·2	51·4	64·5
50	D4ER	50·6	51·3	65·5
55	AN_5	49·3	47·7	68·6
56	AN_5	49·3	—	66·9
67	C	—	55·0	60·6
68	C	—	54·7	56·2
70	D4ER	52·0	51·3	71·2
71	D4ER	55·3	55·4	65·7
76	C	—	54·7	56·2
77	C	—	55·5	58·8
85	Otto	54·5	—	80·1
86	Otto	54·8	—	77·9
94	RLE	33·5	—	76·7
95	RLE	34·2	—	77·1
97	Teps	33·7	—	74·0
98	Teps	32·3	—	75·3
115	Fortuitum	—	63·7	81·2
116	Fortuitum	—	61·6	82·4
122	CB17	42·2	—	82·7

TABLE 7

CRUDE PHOSPHATIDE PHOSPHORUS CONTENT OF SOME ACTIVE AND LESS ACTIVE FREEZE-DRIED ANTIGENS

Antigen No.	*Strain from which antigen prepared*	*mg. lipid per 100 mg. antigen (Et_2O-EtOH) soluble*	*mg. crude phosphatide per 100 mg. antigen*	*µg. crude phosphatide phosphorus per 100 mg. antigen*	*C.F. activity* µg. dry wt. antigen at end-point*
"Less active"					
49	D4ER	44·8	19·6	10·0	7·2
50	D4ER	39·8	19·4	20·4	9·6
55	AN_5	39·2	13·0	35·7	10·2
56	AN_5	39·5	9·7	39·1	10·8
"Active"					
94	RLE	32·1	15·4	198·0	0·35
95	RLE	32·6	17·4	245·3	0·35
97	Teps	32·5	10·1	119·2	0·50
98	Teps	31·0	17·7	198·8	0·25

* against anti-bovine serum.

Examination of 20 of the antigens showed that the proportion of UAF approximated to the amount of methanol or alcohol-soluble material extractable from the antigens, suggesting the UAF to be lipid in nature and possibly therefore related to the methanol antigen of Boquet and Nègre (Table 6). Further confirmation was obtained by analysis of 4 active and 4 relatively inactive preparations, which showed that activity was related to their content of crude phosphatide phosphorus, i.e. lipid material insoluble in acetone (Anderson, 1936). The relationship is a quantitative one and again apparently independent of type (Table 7).

Many other experiments were carried out on miscellaneous preparations and will not be reported here in detail. For example, the dried aqueous extracts further extracted with methanol lost all serological activity, which now appeared in the methanol. Methanol extracts of acetone and water extracted bacilli i.e. those from which Maltaner-Wadsworth extracts had been prepared, yielded large amounts of active material and no further activity could be extracted from the bacillary residues by a second boiling with water. There is, therefore, good evidence that the active fractions of methanol and aqueous extracts are similar and that activity is related to the content of crude phosphatide. Serological activity is not, however, a simple function of the phosphatide. Phosphatides prepared from crude PPD, methanol extracts and aqueous extracts exhibited activity which diminished with purification, and it seems likely that, arguing by analogy with the Wassermann antigen (Pangborn, 1949), the wax of the bacillus plays a role in the manifestation of activity.

The active material in aqueous extracts does not pass through "Visking" cellophane membranes with a pore size of about 20 mμ during dialysis, or after ultrafiltration.

DISCUSSION

All fractions and antigens have been tested against single stock avian, bovine and Johne antisera, all known to contain a high titre of complement-fixing antibodies, and it has been assumed that the titre of the antigen in presence of excess antiserum is a measure of its activity. Such an assumption is justified by the work of de Almeida (1956) on iso-fixation curves, in which he demonstrated that for given levels of complement, maximum antigenic activity was exhibited in presence of excess serum. Our own studies of selected antigens by "checkerboard" methods, confirms this, and it is certainly a useful first approximation in screening tests of a large number of preparations and fractions. It is difficult to define what is meant by the specificity of a mycobacterial antigen. For example, if an active antigen be prepared from *M. johnei*, it can differentiate between cattle infected with Johne's disease and those exhibiting reactivity to avian tuberculin since the latter give

negative and the former positive reactions. The antigen is not exhibiting specificity in the sense of differentiating between mycobacterial antibodies, but is detecting a difference in the nature of the infections i.e. between a moderate pathogen which has not provoked antibody production and a strong pathogen which has. The antigen would react with sera from the rare generalized cases of avian tuberculosis (Jensen, 1956) and with at least 50 per cent of sera from cases of bovine tuberculosis. (Hole, 1956). It is probably this difference in antibody production which permits the effective application of complement fixation tests in the diagnosis of Johne's disease, particularly in the absence of tuberculosis (Bang, 1913) quoted by Sigurdsson (1956)). The problem therefore resolves itself into two parts, firstly, whether an antimycobacterial titre is produced or not, and, secondly, if a titre is obtained, by which mycobacterium it is produced. Since all three sera used in the present experiments were of high titre it is the second point which has been studied here. With the possible exception of antigens prepared from bovine types of organisms it is clear that none of the antigens could differentiate the types of antibody in the three sera. Even with active preparations of the bovine type it is doubtful if differentiation could be made with the varying antibody levels encountered under field conditions. Antigens, whether prepared successively from a single strain or several strains of the same type exhibited a wide variation in activity. The most active antigen obtained was from the human type strain C, but antigens from *M. johnei* and saprophytic strains also exhibited a wide antigenic coverage. There is little evidence of any antigen specificity, the highest titres being obtained with the serum of highest antibody content and with antigens of the highest activity, irrespective of source.

Quantitative chemical examination of the extracts yielded little information of direct value. The preliminary extraction with acetone yielded amounts of serologically inactive lipid which varied widely both within and between types of mycobacteria. The pathogenic types generally yielded more acetone soluble lipid than the saprophytic, but individual strains, e.g. the bovine type CB17, could also give a low yield. An unexpected result was the rather high lipid content of the *M. johnei* strain 3 and 5 Path. Aqueous extracts also varied widely and in a non-characteristic manner. Strains grown on glycerol-beef-broth gave very poor growths of organisms but yielded aqueous extracts qualitatively different from extracts of organisms grown on synthetic medium in that 50 per cent of the material could be centrifuged from suspension. Neither the chemical composition nor serological activity differed widely from synthetic medium preparations.

Linking chemical composition with serological activity revealed a clear correlation with the predominantly lipid portion of the powders, and further, with the content of acetone-insoluble lipid, suggesting that, in common with the methanolic antigens, the active

principle is the phosphatide fraction of the bacillus or is closely associated with it. These findings are very similar to those reported by Pangborn working with the human types of tubercle bacilli and suggest, in addition, that there is a close similarity in the chemical composition of the active antigen in the various types of mycobacteria. The widely varying composition and activity of the aqueous extracts renders them rather unsuitable for finer chemical and specificity studies, but the investigation suggests that preparations of pure phosphatides or fractions of phosphatides, possibly in combination with cholesterol, or tubercle wax and lecithin to diminish anticomplementary effects might give reproducible preparations of antigens. Further work, not reported in detail here, has shown that methanol extracts from which anticomplementary wax has been largely eliminated by extracting at 4°C are rather better antigens in both yield and activity than those prepared by the classical method at 37°C. Phosphatide from such a preparation has lower activity than the original whole antigen, suggesting that the residual wax, which is by itself inactive (Maclay and Paterson, unpublished), potentiates the activity of the phosphatide. Unlike tubercle antigens with syphilitic sera, Wassermann (cardiolipin) antigens give a very low titre with bovine or johne antisera, which suggests the antibody present is antibacterial rather than being produced against an altered tissue component. Nevertheless, studies similar to that of Pangborn and colleagues using various tubercle phosphatides in place of cardiolipin might yield useful antigens and permit critical studies on the specificity of mycobacterial phosphatides.

CONCLUSIONS

The complement-fixing capacity of aqueous extracts of acetone-extracted mycobacteria varied between the mycobacterial types and between successive preparations of the same type.

No evidence could be obtained of serological type specificity of aqueous antigen extracts when tested against experimentally produced anti-mycobacterial bovine sera.

No correlation was found between serological activity and the content of total nitrogen, protein nitrogen, phosphorus or carbohydrate.

A correlation existed between the residual lipoidal material of antigens and serological activity; the activity was independent of the type of mycobacterium from which the extract was derived.

Examination of 4 active and 4 relatively inactive materials showed that activity was related quantitatively to their content of crude phosphatide phosphorus, and was again independent of mycobacterial type.

The nature of the active principle is discussed.

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THE PROPAGATION OF CANINE DISTEMPER VIRUS IN TISSUE CULTURE

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INTRODUCTION

The first accounts of the behaviour of distemper virus in tissue culture come from Mitscherlich (1938) who reported the survival of virulent virus for several days in single-drop cultures of fragments of dog spleen and lung. Little further was done until 1951 when Dedié and Klapotke (1951) confirmed the previous findings using minced suspended tissues from the spleen, lung, lymph node and testis of the dog. The following year these two workers (Dedié and Klapotke, 1952), using the same culture system, maintained growth of distemper virus for a period of 73 and 38 days in dog testis and spleen respectively and for 20 days in dog lung. No further work has been done on the growth of this virus in tissue culture from normally susceptible hosts, and experiments using tissues from an even more susceptible host—the ferret—have not been reported.

Propagation of the virus in normally insusceptible animals by the intracerebral route has been achieved in the case of the suckling mouse (Morse, Chow and Brandly, 1953) and suckling hamster (Gutierrez and Gorham, 1955; Cabasso, Douglas, Stebbins and Cox, 1955), using strains first adapted to the chorio-allantoic membrane of the developing chick embryo.

This communication describes the behaviour of both virulent and adapted virus in roller cultures of tissues from various animals. In view of the remarkable finding that human serum from adults invariably contains antibodies to distemper virus (Imagawa, Yoshimori and Adams, 1953; Imagawa, Yoshimori, Wright and Adams, 1954; Karzon, 1955), both normal and neoplastic human cells have also been investigated.

MATERIALS AND METHODS

Egg-adapted virus. The strain* was egg-adapted and had been passaged many times on the chorio-allantoic membrane. It was completely non-virulent to dogs and ferrets although retaining its capacity to immunize these animals against virulent distemper. Fourteen passages were made in this laboratory before use.

Virulent distemper virus. This strain† was obtained by transference of typical dog distemper infection to ferrets, and has been maintained by serial passage in ferrets. In these animals it gives uniformly 100 per cent mortality.

Titration of virus. Seven or eight-day old eggs were inoculated on the chorio-allantoic membrane as described by Beveridge and Burnet (1946).

* Kindly supplied by Mr. A. B. MacIntyre, Wellcome Research Labs., Beckenham, Kent.

† Kindly supplied by Mr. W. Mansi, Veterinary Laboratory, Ministry of Agriculture, Weybridge, Surrey.

Decimal dilutions of the material were prepared and 4 to 6 eggs used for each dilution. An inoculum of 0·1 ml. was given and the eggs were then incubated at 36° to 37°C. After 7 days the membranes were removed and examined for the presence of lesions, the 50 per cent infectivity end-point being calculated by the method of Reed and Muench (1938). As has been found by others, the linear coalescent lesions which were invariably present made the customary pock-counting method of titration inapplicable.

Source of ferrets. All ferrets used for testing tissue culture material for the presence of virulent virus came from a colony maintained for 25 years. When required for use they were transferred to a specially designed building and placed alone in small cubicles. Full precautions were taken to maintain the strictest possible isolation between ferrets.

Ferret spleen and kidney cultures. Spleens and kidneys were obtained from 1 to 8 week old ferrets, bled out after cervical dislocation, and plasma-clot roller tubes set up. Only the cortical tissue of the kidneys was used for preparing the cultures and the initial medium for both tissues was as follows:

10-15 per cent horse serum inactivated at 56°C for 30 minutes, 5 per cent chick embryo extract, 80-85 per cent mixture 199 (Morgan, Morton and Parker, 1950), 0·1 mg/ml. soya bean trypsin inhibitor (Robbins, Weller and Enders, 1952), 0·01 mg/ml. phenol red, 100 units/ml. penicillin, 100 μg/ml. streptomycin.

The medium was changed twice weekly until epithelial outgrowth was present in addition to the usual fibroblastic cells. This took usually about 6 to 8 days and the medium was then changed to the following: 3 per cent inactivated horse serum, 5 per cent chick embryo extract, 46 per cent bovine amniotic fluid (Enders, 1953), 46 per cent mixture 199. trypsin inhibitor, phenol red, penicillin and streptomycin at the concentrations given before.

The cultures were finally inoculated with virus and the medium changed every 3 or 4 days. Never less than 8 roller tubes in a batch were infected at a time.

Harvesting of ferret cultures. After a period of infection lasting usually about 2 weeks the fluid phase was removed and the tissue rinsed once with Geys' solution (Gey and Gey, 1936). The culture was then scraped off the walls of the tube into an equivalent volume of 0·5 per cent bovine albumin in mixture 199 and ground in a Ten Broeck tissue grinder. After clarification at 1000 r.p.m. for 5 min. the supernatant fluid together with the fluid phase was stored at −76°C until tested for virus a few days later. Passages to other cultures were made with a dilution of 1:10 or greater of the solid phase harvest.

Dog lung and kidney cultures. Animals were killed by cerebral destruction and plasma-clot cultures set up using 8 to 10 explants per tube. Pulmonary tissue was obtained well away from the pleural surfaces of the lobes. The medium of Enders (1953) was used with the addition of 0·1 mg/ml. soya bean trypsin inhibitor and 1·0 ml. was added to each roller tube. Eight tubes or more were used for each passage and changes of medium were made twice weekly. Ten to eleven days after infection the tissue was ground in the fluid phase and after clarification the supernatant fluid used at a 1:20 dilution to infect new cultures.

Cultivation of ferret trachea and rabbit tissue. The trachea of adult ferrets,

killed by concussion and bleeding out from the heart, was removed in toto and opened out by cutting down the ventral aspect. The tissue was then placed in bovine amniotic fluid and left at room temperature for about 2 hours. This enabled the mucous membrane to be subsequently dissected from the cartilage in pieces of a reasonable size using fine cataract knives. Cultures of the columnar epithelium were set up in roller tubes and supplied twice weekly with Enders medium containing 0·5 per cent lactalbumin hydrolysate, trypsin inhibitor and antibiotics. Cultures were harvested by grinding the tissue in the fluid phase and passages made with clarified supernatant fluid at a dilution of 1:20.

Rabbit kidney roller cultures from 7 to 10 day old suckling rabbits were prepared along the lines previously indicated for similar tissue. The medium used was that given above and cultures were harvested after 10 to 11 days.

Human cell line cultures. HeLa cells, human liver* and kidney cells* (Chang, 1954) were all propagated and maintained along the lines described for HeLa cells by Pereira and Kelly (1957) with the following modifications. HeLa cell cultures were inoculated with 20-30,000 cells per tube, and were not infected until after 4 days growth. Human liver and kidney cells were seeded out at a concentration of 100,000 cells per tube. All cultures were given a change of fluid twice weekly and harvests were made by grinding the cells in the medium and clarifying.

Culture medium constituents. The batches of rabbit and horse serum and bovine amniotic fluid used were not found to possess any neutralizing activity against distemper at a dilution of 1/4.

RESULTS

Virus Growth in Cultures of Ferret Kidney

Egg-adapted distemper virus was propagated in roller tube cultures of suckling ferret kidney through a series of 7 passages. The initial material showed a titre of $10^{4.3}$ EID_{50}/ml. and the final harvest a titre of $10^{2.3}$ EID_{50}/ml., the virus being diluted 10^{10} times during the series (Table 1). The period of cultivation lasted a total of 95 days and during the first transference from the 5th to the 6th serial culture the virus was lost. This may have been due to the fact that a trial was made of a simpler maintenance solution containing medium 199 without serum. This medium however was not satisfactory and the cultures showed an increased number of degenerate cells after a few days.

Virulent distemper virus obtained from the spleen of an infected ferret was also passaged in the same type of culture and the harvested tissues tested for activity by intraperitoneal inoculation of susceptible ferrets. Virus was present in the cultures up the 4th passage—a total of 49 days—and gave rise to the typical picture of distemper in ferrets, with appearance of high fever (104° to 105°F.) by the 10th day followed by death. Virus was not recovered from the 5th passage and neither modified disease nor pyrexia occurred (Table

* Kindly supplied by Dr. J. C. N. Westwood, Microbiological Research Establishment, Porton, Wilts.

2). The ferret inoculated also showed no immunity when challenged with virulent virus.

TABLE 1

PASSAGE OF EGG-ADAPTED DISTEMPER VIRUS IN ROLLER CULTURES OF SUCKLING FERRET KIDNEY

Passage	*Log EID_{50}/ml in inoculum*	*Log EID_{50}/ml in final harvest**	*Total period of cultivation in days*	*Dilution of original virus suspension*
1	4·3	4·0	18	—
2	2·7	4·2	39	10^{-3}
3	3·2	4·75	52	10^{-4}
4	3·75	3·5	66	10^{-6}
5	2·5	3·25	77	10^{-7}
6	2·25	2·4	87	10^{-9}
7	1·4	2·3	95	10^{-10}

* washed ground solid phase of culture only.

Growth of Virulent and Adapted Virus in other Ferret Tissues

Virulent distemper virus was passaged through a series of roller tube cultures of suckling ferret spleen and the tissues harvested at fortnightly intervals. The 3rd serial culture was tested for virus after a total of 40 days of cultivation, but no disease or pyrexial reaction was observed in the ferret used. When egg-adapted virus was tested in the same tissue culture system using an inoculum of $10^{4·0}$ EID_{50}/ml., virus was present in a titre of $10^{2·6}$ EID_{50}/ml., when the 3rd passage was harvested at the 38th day and tested on the chorio-allantoic membrane of eggs. No virus was recovered from the 4th passage when harvested 14 days later and this passage was repeated again without success.

In view of the very high susceptibility of epithelium of the respiratory tract to infection in the natural disease in ferrets, an investigation of the behaviour of respiratory tissue was made in vitro. Explants of ferret lung tissue were used first of all, but the outgrowth from most fragments consisted almost entirely of fibroblastic tissue and epithelial cells were very few in number. Cultures of fragments of trachea from suckling ferrets were next tried, but the cellular sheet was very slow in appearing and the extent of growth too variable. Attempts were then made to separate the actual columnar epithelium from the surface of the cartilage of the trachea. Although attached by exceptionally binding fibro-elastic areolar tissue, separation without too much damage to the cellular layer

was eventually achieved and good sheets of epithelium were obtained in vitro. Using cultures 7 or more days old, passages of virus were made and after a total of 26 days of cultivation the 2nd serial harvest was tested in a pair of ferrets housed in the same cubicle. Both showed the fully developed disease within 10 days and were dead on the 12th day.

No degenerative histological changes were observed in the infected cultures, but small numbers of multinucleate giant cells developed during the last few days of cultivation. These were not observed in the control cultures. An appreciable degree of cellular differentiation must have occurred at places in the epithelial sheet as islands of ciliated columnar epithelium were present well away from the original explant. These cilia maintained their normal rate of motility throughout the period of infection.

TABLE 2

PASSAGE OF VIRULENT DISTEMPER VIRUS IN SUCKLING FERRET KIDNEY CULTURES

Passage	*Total period of cultivation in days*	*Virulence for ferrets*	*Dilution of original virus suspension*
1	17	NT	—
2	31	NT	10^{-2}
3	42	+	10^{-3}
4	49	+	10^{-5}
5	63	0	10^{-6}

NT = Not tested.

Behaviour of Virulent Distemper Virus in Cultures of Dog Tissue

Serial virus cultivation was made for 30 days in roller tube cultures of kidneys taken from two dogs of about 7 to 9 years old. It was found that the renal tissue from these rather old animals required at least 12 days cultivation before the cells were ready for inoculation. Three passages were made and the final harvest tested in two ferrets by intraperitoneal inoculation. Neither ferret showed evidence of disease and when challenged subsequently with virulent virus one died on the 13th day and the other was killed 2 days later when seriously ill. The growth of egg-adapted virus was not investigated in dog tissue cultures.

Preparations of lung tissue taken from the same two dogs were also inoculated with a similar quantity of virulent virus. These cultures, in contrast to ferret lung, had developed good zones of epithelium by the 12th day when virus was added. No specific cytopathological changes were detected and the small numbers of

degenerate cells which occurred were present in approximately equal numbers in both test and control tubes. After a total of 30 days' propagation the 3rd serial culture was harvested and tested in two ferrets. Both developed distemper on the 7th day beginning with anorexia and fever; by the 14th day one was moribund and the other had died.

Distemper Virus in Rabbit Kidney Cultures

Three serial cultures of virus were made in suckling rabbit kidney roller tubes at 10 to 11 day intervals. When the final harvest of tissue and fluid was made the total period of cultivation was 31 days, and this material was tested intraperitoneally in one ferret which showed a sudden rise of temperature to 104° to 105°F. a week after inoculation, followed by gradual recovery. On subsequent challenge with virulent virus this animal showed no evidence of immunity, developing typical distemper after the usual incubation period.

Distemper Virus in Human Cells

Groups of cultures of HeLa cells, human liver and human kidney cells were tested for the capacity to support multiplication of virulent distemper virus. Two passages were made in HeLa cells and after a total of 24 days cultivation the ground cells were tested in a ferret. Typical disease was produced with death on the 13th day. Exactly the same quantity of virus inoculated into human kidney cells gave no evidence of the presence of active virus after 2 passages lasting altogether 30 days.

Human liver cells however, when similarly tested after 2 passages, induced conjunctivitis starting on the 9th day followed by fever, lesions on the mucous membranes and death on the 15th day. As no cytopathological changes were observed in cultures of any of the three cell lines, and stocks of susceptible ferrets were exhausted for the season, further studies with the unadapted strain of virulent virus were discontinued.

Summary of Experiments

The activity of two strains of distemper virus in cultures of various tissues is shown in Table 3. The growth of egg-adapted distemper was maintained for 95 days through 7 passages in ferret kidney roller cultures, and for 38 days through 3 passages in ferret spleen cultures. In the former cultures virulent virus was maintained for 4 passages totalling 49 days and then lost, but it was not recoverable after 40 days in the latter cultures.

DISCUSSION

All attempts at continuous propagation of distemper virus in normally insusceptible hosts were a failure until Haigh succeeded in adapting the virus to the chorio-allantoic membrane of eggs (Haigh, 1948 a,b.), using a strain attenuated by prolonged passage

TABLE 3

Animal species	*Cell type*	*Virulent virus*			*Egg-adapted virus*		
		No. of passages	*Period of cultivation in days*	*Presence of virus*	*No. of passages*	*Period of cultivation in days*	*Presence of virus*
FERRET ..	Kidney	4	49	+	7	95	+
„ ..	Spleen	3	40	—	3	38	+
„ ..	Trachea	2	26	+			
DOG ..	Kidney	3	30	—			
„ ..	Lung	3	30	+			
RABBIT ..	Kidney	3	31	—			
HUMAN ..	Kidney	2	30	—			
„ ..	Liver	2	24	+			
„ ..	HeLa	2	24	+			

in ferrets by Green (1939), and further examined by Green and Swale (1939).

Using such an egg-adapted strain, propagation of the virus has been achieved for a period of 95 days in suckling ferret kidney tissue culture. Titres of virus recoverable have not been as high as those which can commonly be obtained in eggs, but observations on the best time of harvesting etc., have not been made. Serial propagation of the fully virulent strain in these cultures was not successful however, and this disparity between adapted and unadapted strains was also apparent in ferret spleen cultures. Despite the behaviour of ferret kidney, cultures of dog kidney were not found to support growth of virulent unadapted virus. This finding is in keeping with the observations of Dedié and Klapötke (1951) who succeeded in passaging the virus for a time in cultures from 4 different organs of the dog, but were unsuccessful when using kidney tissue. Failure may however be due to using tissue from old dogs where the capacity for cellular proliferation and dedifferentiation is presumably much less, and the kidney tissue is to some extent sclerotic.

Although no pathological changes of the more common degenerative kind were found in any of the cultures supporting virus multiplication, changes were noticed in the respiratory epithelium in ferret though not in dog cultures. They were observed by low power microscopy and consisted of the appearance of a small number of giant cells containing 3 to 5 nuclei. Unfortunately no staining was done to determine whether such cells actually contained inclusion bodies. Adams and Imagawa (1955) have reported finding typical destruction of HeLa cells during the first 2 passages of distemper virus with subsequent failure to achieve serial cultivation. Only limited tests have been made with virulent distemper, but no abnormal changes have been observed in any of the human cell cultures infected.

The significance of the presence of virus in cultures where only 2 or 3 serial passages have been undertaken must be interpreted in the light of the survival of virus at incubator temperature (37°C). Celiker and Gillespie (1954) studied the survival of distemper virus suspensions under various conditions and found that complete loss of activity occurred after 8 days at only 25°C, while at 50°C inactivation was effective in 1 hour. Haigh (1956) also found a heavy fall in infectivity from 10^{-4} to less than 10^{-1} after 48 hours at the lower temperature of 32° C. The recovery of virus from serial cultures after intervals greatly in excess of this inactivation period would appear to indicate that virus multiplication is occurring. Another possible explanation, however, could be that the thermal inactivation of virus is somehow interfered with by the presence of living cells.

CONCLUSIONS

Kidney cells from suckling ferrets were found to provide a

suitable environment for serial growth of egg-adapted distemper. Unadapted virus will grow only for a limited time in these cultures, and not in dog, rabbit or human kidney cells. Spleen cultures will not support continuous virus multiplication, but respiratory tissues of the ferret and dog may do.

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INFECTIOUS GASTRO-ENTERITIS OF PIGS

I. The Disease in the Field

By

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INTRODUCTION

In 1957, we encountered a highly infectious gastro-enteritis of pigs in East Anglia (Goodwin and Jennings, 1958a) and a brief description has already been given of the main clinical and post-mortem findings (Goodwin and Jennings, 1958b). The present paper enlarges upon the earlier reports and brings together information on the clinical signs, epidemiology, gross pathology and histology of the disease as it occurs in the field.

RESULTS

Clinical Observations

Ten major outbreaks were studied in herds where the number of breeding sows and gilts ranged from twenty-three to about sixty. In all, one hundred and thirty-seven litters of suckling pigs were affected and, from these, six hundred and ten piglets died.

The Disease in the Individual

Adult pigs. Adult pigs were often badly affected; they developed an acute diarrhoea, commonly after showing partial or complete anorexia the previous day, but sometimes without any warning of impending illness. The diarrhoea was usually grey or brown, rather than yellow, and in many cases it squirted from the rectum like a jet of water. Animals with such marked diarrhoea, however, showed a wide variation in their general condition. Some appeared to lose little weight, whereas others became thin and weak; they could then hardly walk and they stood swaying slightly with the hind legs crossed. There was often a marked thirst. A few sows had a watery ocular discharge early in the infection and vomiting was another infrequent sign. Less severely affected animals had loose faeces and appeared dull. The looseness of the faeces was often the only sign that they were undergoing infection and there were many instances where it was difficult to know whether a sow had experienced the disease or not. The extent to which subclinical infection occurred is a matter for further investigation. Most owners commented on the markedly offensive smell of the diarrhoea and claimed that it was peculiar to this disease. Almost without exception there was no significant rise in the rectal temperature. Nervous signs were absent.

In sows there seemed to be an important correlation between

the onset of the disease and farrowing. Thus many sows were not affected until a day or so before, or shortly after farrowing, and it was a common sight to see the sow and litter affected together about the 2nd or 3rd day post partum. Although it must be borne in mind that sows are commonly brought into the farrowing house about 1 week before parturition is due, and that the farrowing house could be a more intense source of infection, it was remarkable that on two farms groups of pregnant sows, close to the farrowing house and fed by the same man, remained free of the disease. Evidence was obtained on other farms that sows affected while pregnant often had the disease only mildly, and it is possible that some of these pregnant sows had actually suffered a sub-clinical infection: this cannot be the whole explanation, however, for the disease continued to affect the sows in turn as their farrowing day arrived. This tendency of the pregnant sow to withstand infection and then break down subsequently at parturition could contribute to the seriousness of the disease. In the lactating sow, partial agalactia was common; the udder appeared to contract and the skin over the mammary glands became wrinkled. In sows about to farrow this clinical sign, instead of the usual rapid pre-parturient expansion of the udder, was found by one owner to be a good indication that a sow would be affected at or after farrowing.

Only three adult animals died, always from a very acute attack of the disease; in one instance this was associated with an intussusception. There were no deaths from chronic weakness or debilitation and there were no other ill-effects, such as abortions or still-births. Most badly-affected animals were beginning to eat by the 4th to 7th day after the onset of diarrhoea and anorexia.

Previous vaccination against swine fever conferred no immunity to this type of gastro-enteritis.

Fattening pigs. The disease in weaners and fattening pigs behaved almost exactly as in the adult stock. Sometimes the diarrhoea was profuse and at other times barely loose. The recovery time was the same. Although many bacon pigs looked obviously ill and dejected, most showed surprisingly little over-all effect of the profuse diarrhoea and the main importance of the disease lay in its retardation of the growth rate. Table 1 shows the average weight gain of twenty pigs that were affected together in a fattening house. Although the disease did not arise simultaneously in all the pigs, most started to scour about Jan. 24th and recovery was almost complete by Jan. 30th and 31st. It can be seen that the average gain in weight during the 9 days before infection was 8 lb.; thereafter, there was no mean gain in weight until after recovery and the economic effect in this instance, therefore, was equivalent to a standstill in growth during the period of clinical signs.

Suckling pigs. In the suckling pig the main sign was, once more, diarrhoea, but it was usually much lighter in colour. Not uncom-

monly the diarrhoea had a greenish tinge. As in the adult, there were no nervous signs, except in the terminal stages when some piglets exhibited a rigidity and a slight tendency to torticollis just before death, nor was there pyrexia. Vomiting was not uncommon, but it did not occur repeatedly in any individual piglet.

TABLE 1

EFFECT OF INFECTION ON GROWTH

Average weights of a group of twenty fattening pigs during the period of infection.

Weighing date	*Jan. 11th*	*Jan. 20th*	*Jan. 27th*	*Feb. 3rd*
Average weight (lb.)	157	165	165	169

Most of the pigs started to scour about Jan. 24th and recovery was almost complete by Jan. 30th or 31st.

The dominant factor in the piglet was the age at first infection and this above all else determined the form and outcome of the disease. Thus new-born piglets died within 2 days of first showing diarrhoea, whereas pigs between 1 and 2 weeks of age often scoured for several days before dying. In one herd, where some litters were nearly 3 weeks old at the first signs of diarrhoea, deaths continued to occur up to 17 days later. Piglets over 3 weeks of age at first infection showed a temporary check in growth, which was more marked the younger the animal. Table 2 shows the mortality rate at different ages taking only those litters for which the information is reliable. The small numbers in the groups over 3 weeks of age are due mainly to two factors. First, less note was taken by owners when all the litters on a farm were over this age and, secondly, the natural spread of the disease in successive litters of new-born pigs swells the totals at the younger ages. The mortality for each age group will be considerably influenced by any concentration of piglets at either end of the range, there being a great difference, for example, between the effects of the disease at 8 days compared with 14 days. Nevertheless, the table allows broad conclusions and demonstrates well the marked improvement in the recovery rate with each week of life.

Several farmers maintained that the chances of survival could be measurably improved by early weaning. Whether this was due to improved nutrition, the solid diet, or other factors is not clear.

The Disease in the Herd

In every herd the source of the disease was obscure and although in most herds there were many possibilities of indirect contact, such as from food lorries or other transport, no obvious contact with the disease elsewhere could be described and there had been no recent

importation of pigs on any of the farms. On one farm, the original herd had been sold in March and the yards had been free of pigs until July, when a new herd had been established from premises where the disease has yet to appear. The new herd was kept in strict isolation, but despite the use of their own lorries and food sacks and the absence of any known contact with the disease, the herd became affected in January. It would seem, therefore, that unknown indirect contacts were the common means of introducing infection.

TABLE 2

EFFECT OF AGE ON MORTALITY

Age at start of diarrhoea (days)	*Piglets affected*	*Piglets died*	*Mortality (%)*
0—7	349	319	91
8—14	188	97	52
15—21	142	36	25
22—28	23	2	9
29—35	27	0	0

The first case of the disease in each herd was never among very young piglets. More commonly, the earliest sign of inappetance and diarrhoea was in the adult stock, but in three herds the disease first appeared in pigs between 5 and 8 weeks of age. The progress of the disease varied thereafter but, in general, the spread was rapid. In the most acute outbreak seen, involving a herd of twenty-three sows, nine litters and nearly one hundred fattening pigs, all the animals became affected within 7 days of the first case. In other herds, however, the infection was not universal and large groups of animals appeared to escape the disease. Thus in one large fattening house only an occasional pig scoured, even though the young litters a short distance away were being decimated. Once the disease had spread throughout such of the fattening and adult stock as seemed susceptible, the subsequent progress was governed largely by the farrowing time-table. The successive infection of young litters and often of their dams at parturition prolonged the course of the disease considerably; in one herd, for example, the disease was maintained in this way for 11 weeks in a series of thirty-five litters. Conversely, a

natural break in the farrowing sequence could bring an outbreak to an end, even though the sows farrowing after the break had often not appeared to have had the disease earlier. The durations of these effective breaks, where they occurred, were 6, 7, 2 and 7 weeks. The intervals quoted are those between the onset of the last clinical case and the date of birth of the next litter.

Haematology

Blood samples were taken on two farms from five pigs in various stages of the disease. The haematological findings are shown in Table 3. Only the leucocyte counts are given and these show a marked mononuclear reaction.

Pathology

Morbid Anatomy

The account that follows is based entirely on the disease in the unweaned piglet. The extent and nature of the gross lesions varied directly with age. Thus in the very young piglet, where death occurred in the first few days of life, there were often no lesions; on the other hand, pigs dying during the third week of life or later showed severe lesions, including ulcerative gastritis.

External appearance. Many of the older piglets were in good condition and showed no signs of dehydration. New-born animals were commonly in poorer condition but, even so, they were not often dehydrated, presumably because death occurred so rapidly at this age. The skin, particularly in young piglets, was commonly an unnatural strong-pink colour, especially on the abdomen, the neck region and the base of the ears. The hindquarters were sometimes soiled with diarrhoea.

Stomach. The stomach was normal in about half the cases, mainly in the very young pig. Otherwise, there were all degrees of change, ranging from a few pin-point petechiae in the pyloric area to acute fibrinous inflammation and extensive ulceration (Fig. 1). The gastritis was sometimes seen from the serous surface as an area of dark discolouration, but in other cases it was not apparent until the stomach contents, usually a hard stringy curd, had been completely washed away. In some cases of haemorrhagic gastritis the curd was pink in colour. The mucous membrane was often covered with a grey fibrinous deposit beneath which were ulcers or haemorrhages. The ulcers, which were not confined to the pylorus, had raised edges, showed no signs of healing and did not extend into the muscle coats. They were not seen in pigs younger than 2 weeks of age but, over this age, they occurred in 10 per cent of the cases.

Small intestine. Once again, the very young pig commonly showed no abnormality of the intestine, except for a striking distension of the jejunum and ileum with gas (Fig. 2), and a catarrhal enteritis. In about 25 per cent of cases the enteritis was haemorrhagic, the red-

TABLE 3

LEUCOCYTE COUNTS IN AFFECTED PIGS

Pig	*Time from onset of diarrhoea*	*Leucocytes (c.mm.)*	*Unseg. neutrophils* (%)	(c.mm.)	*Seg. neutrophils* (%)	(c.mm.)	*Eosinophils* (%)	(c.mm.)	*Basophils* (%)	(c.mm.)	*Lymphocytes* (%)	(c.mm.)	*Monocytes* (%)	(c.mm.)
Bacon pig	12 hrs.	24,000	—	—	29	6,960	5	1,200	—	—	30	7,200	36	8,640
In-pig gilt	24 hrs.	16,000	—	—	60	9,600	5	800	—	—	15	2,400	20	3,200
In-pig sow	24 hrs.	7,000	2	140	12	840	10	700	5	350	36	2,520	35	2,450
Young boar	4 days	21,000	—	—	43	9,030	6	1,260	2	420	14	2,946	35	7,350
In-pig sow	6 days	22,000	—	—	57	12,540	3	660	2	440	11	2,420	27	5,940

ness extending through all the intestinal layers and sometimes colouring the gut contents. Although this more acute type of enteritis was mainly seen in older piglets, it also occurred in the first week of life. In piglets of all ages the gut wall was flaccid; the gut contents were semi-liquid and often yellowish-green. Another constant finding was engorgement of the mesenteric vessels and lymph nodes.

Spleen. In rare instances there were splenic infarcts.

Kidneys. Sub-capsular petechial haemorrhages were sometimes present in the kidney. In young piglets these could not be distinguished from the haemorrhages that are invariably seen post mortem, but they were also seen in pigs over 3 weeks of age, at which time haemorrhages are not normally present. In some cases the kidneys were pale and exceptionally they contained urates.

Urinary bladder. In a few instances among older piglets the bladder showed petechial haemorrhages.

Respiratory tract. In over 75 per cent of piglets the lungs were normal. In a few animals there was a pneumonia of the apical and cardiac lobes, but these came from a herd known to be badly affected with enzootic pneumonia. Petechial haemorrhages were occasionally present on the epiglottis.

Heart. The heart was frequently flabby and appeared grey. Exceptionally, petechiae were seen in the coronary grooves.

Central nervous system. Meningeal congestion with occasional haemorrhages into the membranes, especially into falx cerebri, has been a common finding. The cerebral convolutions were somewhat flattened and moist. In a few cases there were haemorrhages in the cerebellar peduncles.

Histology

Central nervous system. The meningeal congestion seen macroscopically was confirmed histologically: the vessels were packed with red cells and leucocytes, particularly those in the cerebellum (Fig. 3). In three of the older piglets, aged 2 weeks or more, there was evidence of encephalitis. The perivascular cuffing, however, comprised only a single layer of cells and the vascular endothelium showed no proliferation. The dorsal root ganglia and the spinal cord showed no lesions.

Stomach. The only consistent change was acute congestion of all the vessels of the stomach wall. The most marked changes included large areas of necrosis near the pylorus demarcated by dense zones of leucocytes, both round cells and neutrophils. Free red cells were present in the damaged tissue. In less severe cases the surface epithelium showed degenerative changes with fibrin on the surface. In one herd, yeast-like organisms were present on the mucous membrane and also in the crypts.

Small intestine. In almost all cases the vessels of the mucous membrane and the submucosa were very acutely congested, even when there was no other evidence of enteritis. About half the sections examined showed no more than an increased activity of the mucous glands; this was particularly so with material from very young pigs. The earliest degenerative change seen was swelling of the tips of the villi, together with congestion of the subterminal vessels. Subsequently, epithelial cells of the tip were vacuolated and their nuclei had disappeared (Fig. 4). This was followed by necrosis of about half the length of the villus, groups of necrotic villi frequently being surrounded by normal tissue (Fig. 5). Sometimes, however, the necrosis was more widespread, so that only fragments of villi, infiltrated with neutrophils, remained. In the macroscopically haemorrhagic type of enteritis, the mucous membrane was infiltrated with many free red cells, which were also present in the gut lumen. In both types of enteritis there was patchy infiltration of the submucous tissue with round cells.

Large intestine. The changes in the large intestine were less pronounced, but there was some round-cell infiltration, vascular congestion and, rarely, necrosis of the surface epithelium associated with diphtheresis.

Kidneys. About 50 per cent of the kidneys examined showed degenerative changes in the convoluted tubules that had often advanced to necrosis with occlusion of the lumen (Fig. 6); the glomeruli, however, showed no significant changes.

Lymph nodes and spleen. The vessels were congested and there were many free red cells. There was sinus catarrh, the chief infiltrating cell being a macrophage. Many of the cells of the lymph follicles had undergone division and mitotic figures were prominent.

Heart. Despite the gross flabbiness of the heart, there were only slight microscopic changes comprising separation of the muscle bundles, some loss of striation and slight leucocytic infiltration.

DISCUSSION

This disease is now widespread in East Anglia and it has also been seen elsewhere in England. More affected herds could have been examined, therefore, than those described, but by restricting the field investigations in the first instance to ten herds, we were able to study the condition experimentally at the same time. Since, as yet, there is no specific diagnostic test, the diagnosis has been made after considering all the clinical, epidemiological and pathological evidence. The pathological findings will be discussed first.

Although there are no pathognomonic lesions, either gross or microscopic, such as inclusion bodies, certain changes taken together are strongly suggestive of the infection. These are acute meningeal congestion, acute haemorrhagic and sometimes ulcerative gastritis,

and varying degrees of enteritis. Histologically, a striking and consistent finding has been the congestion of all the gastric, intestinal and mesenteric vessels, frequently with diapedesis. Other notable changes included tubular necrosis in the kidneys, the degenerative changes at the tips of the villi and the selective necrosis of groups of villi.

In one outbreak, the presence of petechial haemorrhages in the larynx, bladder, lymph nodes, brain and occasionally in the lung and heart, together with splenic infarcts and slight encephalitis in three animals, suggested swine fever. The piglets showing such changes, however, were mostly over 3 weeks of age and younger piglets dying on the same premises did not show this general haemorrhagic syndrome. In the other outbreaks, most of the dead piglets examined were younger than 3 weeks of age but the few pigs that died at about this age likewise showed widespread petechial haemorrhages. It seems, therefore, that the haemorrhagic syndrome was probably associated with the age at death and the duration of the illness, rather than with the presence of swine fever, and this was supported by the results obtained in the gel-diffusion precipitin tests. Fifteen pancreases were tested from the herd mentioned previously, where some encephalitis had been seen, and all these tests were negative for swine fever. The elimination of Aujeszky's disease as another possibility has been described elsewhere (Goodwin and Jennings, 1958b).

Clinically, a typical outbreak affecting a whole herd is unlikely to be confused with any other known disease. It is unusual for adult pigs to scour profusely, particularly on a herd basis, and the widespread occurrence of diarrhoea in pigs of all ages on a farm, coupled with heavy mortality in the very young piglets is almost diagnostic in itself. Other significant points are the duration of the illness, the occasional vomiting, the normal body temperature, the short incubation period and the rapid spread by indirect contact. When only young litters are affected, however, the entity is less clear-cut and if, as has been seen in the field, most of the piglets are about 3 to 5 weeks old, the disease can simulate the less specific diseases that are commonly associated with diarrhoea at this age. These circumstances might be particularly misleading if the suckling pigs showed the widespread petechial haemorrhages mentioned before and the unaffected sows were vaccinated with crystal violet vaccine; for then the lesions in the piglets and the immunity of the adult stock could seem consistent with a diagnosis of swine fever.

Diarrhoea is not uncommon among new-born pigs and in some herds there is a persistent problem of this type, which is believed to be associated with *E. coli*. Although the onset of this neonatal diarrhoea can occur at about the same time after birth as in the disease that we describe the clinical similarity ends there. Pigs over 1 week of age are rarely affected when neonatal diarrhoea is a problem and the sows suckling such scouring litters are usually

normal; this former condition also responds, in the main, to antibiotic therapy, which appears to have a marked influence on the mortality rate. There may be very little gross pathological change at autopsy, however, in piglets dying shortly after birth from either of these two conditions. These observations on differential diagnosis are based on the overt, acute disease and it is not known whether a less dramatic illness might occur in a population partially immune to infectious gastro-enteritis.

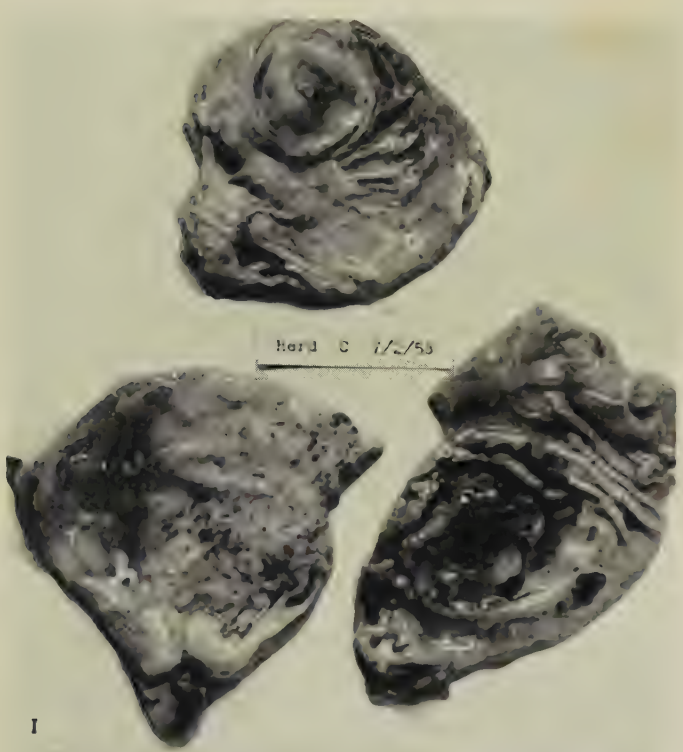
The similarity between the highly infectious gastro-enteritis occurring in England and Transmissible Gastro-enteritis originally described by Doyle and Hutchings (1946) in the United States is, as far as present knowledge allows, virtually complete. According to these authors, the American disease is characterized by diarrhoea in pigs of all ages, with some vomiting and rapid loss of weight. Adult animals almost invariably recover in a few days, but mortality is very high among new-born piglets. There is gastritis and enteritis, engorgement of the mesenteric vessels and albuminous degeneration in the kidney. The hyperaemia and haemorrhage of the stomach and intestine can easily be seen from the serous surfaces and the fluid intestinal contents are sometimes a greenish colour. The highly infective nature of the disease has been stressed (Hutchings, 1947) and an entire herd may be affected within a day or two (Doyle, 1951). As in this country, some outbreaks appear to be limited entirely to the young pigs (Doyle, 1951) and often the infection has no obvious origin (Ray, 1947), although McNutt (1953) did not find this so. Many authors emphasize the marked reduction in mortality with each week of life and the mortality rates summarized earlier in this paper (Table 2) parallel those given in similar tables by Smith (1956). The marked smell of the diarrhoea has been mentioned by Bay, Doyle and Hutchings (1951). The descriptions by the last authors and by Lee, Moro and Baker (1954) of the pathological changes in Transmissible Gastro-enteritis agree closely with our findings. However, the meningeal congestion, commonly seen in our cases, was not cited in these two papers; neither does the haemorrhagic syndrome seen in older piglets receive comment in the American literature.

CONCLUSIONS

A description is given of a highly infectious gastro-enteritis of pigs which is widespread in East Anglia.

Clinically, the disease is highly infectious, has a short incubation period and causes heavy mortality among new-born piglets. There is profuse diarrhoea, often on a herd basis, occasional vomiting, but rarely pyrexia.

The chief lesions are varying degrees of gastro-enteritis, with acute congestion of the mesenteric and meningeal vessels. In new-born piglets the only macroscopic finding may be ballooning of the jejunum with gas.



1

2

Fig. 1. Three stomachs showing fibrinous deposits, gastritis, petechial haemorrhages and ulceration.

Fig. 2. Discoloration of serous surface of stomach, distension of jejunum with gas, and acute congestion of mesenteric vessels.

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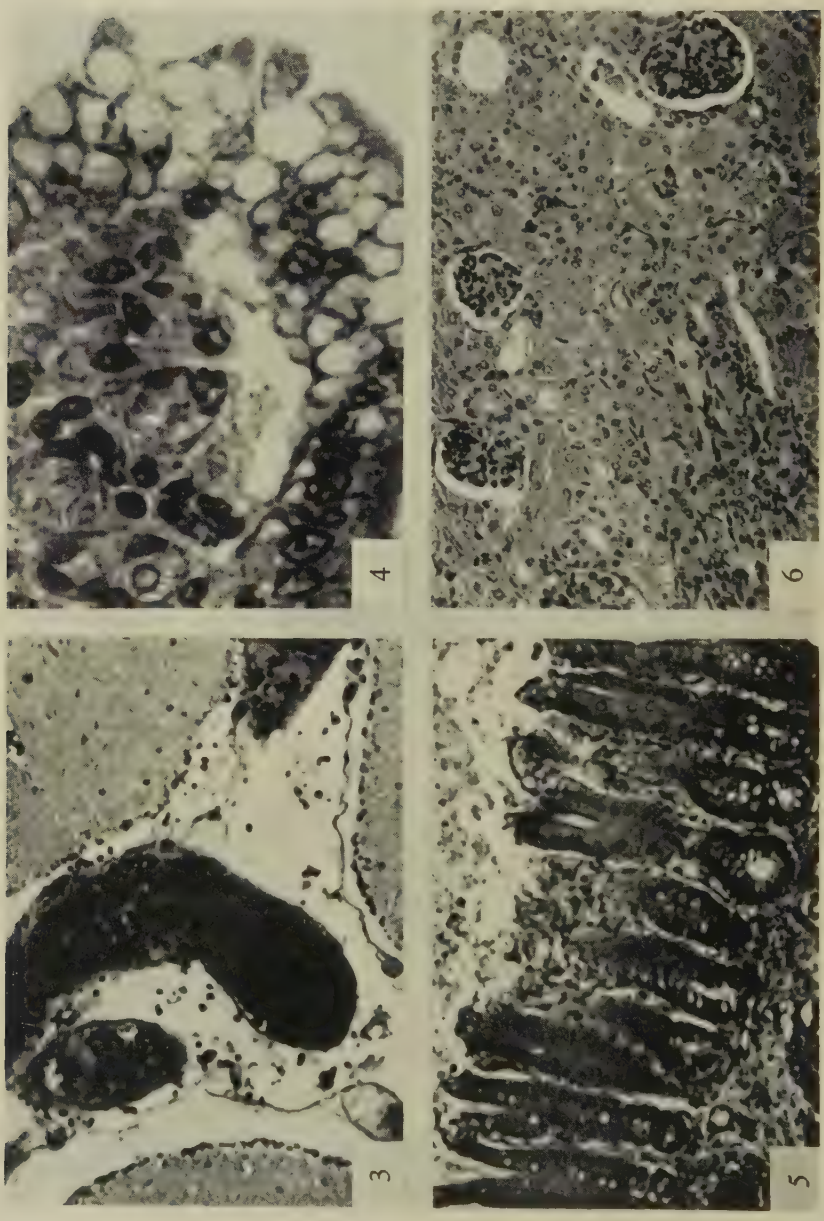

Fig. 3. Sections of cerebellum showing the striking vascular congestion and free red cells. H. & E. × 180.

Fig. 4. Tip of villus showing vacuolation and breakdown of nuclei. H. & E. × 840.

Fig. 5. Small intestine showing selective necrosis of villi. H. & E. × 120.

Fig. 6. Kidney showing tubular necrosis and occlusion of lumina. H. & E. × 180.

The disease closely resembles the Transmissible Gastro-enteritis described in the United States.

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ARTERIOSCLEROSIS IN CERTAIN WILD ANIMALS DYING IN CAPTIVITY

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INTRODUCTION

The observations to be recorded here form part of a study on the effect of captivity on the development of spontaneous aortic arteriosclerosis in mammals and birds. For this purpose aortae were selected from captive animals which at autopsy showed macroscopical abnormalities or were known to be senile. During the course of the study it became obvious that certain aspects of the morphological changes either had only scant attention paid to them or had not been recorded as occurring in animals. This paper records the frequency of the accumulations of mucoid material in animal arteriosclerosis; it considers evidence for the episodic nature of animal arteriosclerosis and also notes certain features peculiar to animal arteriosclerosis.

Many observers believe that accumulation of mucoid material, thought to be acid mucopolysaccharide, in the intima and media precedes and determines the site of intimal lipid deposition in human arteriosclerosis (Moon and Rinehart, 1952 ; Wartman, 1956). Fox (1933 a) in his study of over 10,000 animals observed insignificant mucoid changes in occasional cases of animal arteriosclerosis, and though Rinehart and Greenberg (1951) and Lindsay and Chaikoff (1957) have attached great importance to the accumulation of mucopolysaccharides in the evolution of certain spontaneous and experimentally induced animal lesions, changes of the degree and frequency observed in this study do not seem to be well-known.

Recently Morgan (1956) has shown in a study on the pathogenesis of coronary occlusion that "layering" is common in the atherosclerotic plaque, a finding which he interprets as evidence in favour of the episodic nature of the arterial disease. The aorta was chosen for this study as it is a readily comparable vessel in all animals and in spite of the differences between human coronary arteries and animal aortae some evidence was obtained for the concept that arteriosclerosis in animals is also episodic

MATERIALS AND METHODS

Material was kindly made available by permission of the Prosectorial Committee of the Royal Zoological Society, London. The heart and main arteries were saved from any animals where autopsy showed the presence of arteriosclerosis. In addition certain animals dying of senility or cardiorenal failure were studied, as well as a female lion suffering from muscular dystrophy and killed by order at the age of $3\frac{1}{2}$ years. In all, 40 animals were collected (Table 1).

The macroscopical appearances were noted, following which blocks were taken from any abnormal aortic areas. When naked eye appearances

were normal (in certain animals dying of senility) the junction of the thoracic and abdominal aorta was selected, unless there was apparent difference in thickness between these parts of the aorta; in these cases blocks were taken from both regions. All sections were stained by Haematoxylin and Eosin (H and E), Weigert, or Weigert-van Gieson for elastic tissue, and periodic acid-Schiff (P.A.S.). In addition certain sections were stained by Lillie's buffered toluidine blue method, and in the majority of animals frozen sections were made and stained by Sudan IV for lipids. When gross lesions were present multiple sections were stained.

TABLE 1

OUTLINE OF CHANGES OBSERVED

	Total	*Mammals*	*Birds*
Total number of animals studied	40	7	33
No. showing arterial changes	36	5	31
*No. showing diffuse intimal thickening ..	5	2	3
No. showing focal intimal thickening ..	22	2	20
No. showing focal intimal thickening with intimal fatty accumulation (= atheroma) ..	18	1	17
No. showing medial degeneration alone ..	9	1	8

*This change was not noticeable macroscopically.

RESULTS

The results are recorded in two tables. Table 1 shows the outline of the changes and Table 2 a summary of 20 animals showing moderate or marked changes. The microscopical appearances were defined and graded as follows.

Diffuse intimal thickening. (Fig. 1) A process in which the intima is thickened uniformly by fibrous tissue derived either from its fibrous subendothelial layer, or from the musculo-elastic layer (Fox, 1933b). Changes were graded as follows. Grade 1 (slight) in which there are approximately 3 to 5 cell layers of intimal thickening. Grade 2 (moderate) in which there are more than 5 layers of intimal thickening.

Focal intimal thickening. (Figs. 2, 5, 11) A process in which the intima is thickened focally. Lesions varying from those showing the accumulation of fat or other lipids, to those in which the thickening was due almost entirely to fibrous tissue, were all included in this group. Changes were graded as follows. Grade 1 : intimal thickening which at no point was greater than one third of the thickness of the media of the vessel in an area unaffected by intimal disease. Grade 2 : intimal thickening equivalent to between one third

TABLE 2

SUMMARY OF 20 ANIMALS SHOWING MODERATE OR MARKED CHANGES

	Animal	*D.I.T.*	*F.I.T.*	*I.F.A.*	*M.D.*	*M.M.A.*	*Comment*
Mammals							
	Lion	2	0	0	2	3	See Fig. 1. Mucoid material PAS +ve.
	Korin Gazelle..	0	2	0	3	1	See Figs. 11 and 12.
	Camel	0	3	1	2	1	First time observed in species.
	Zebra	2	0	0	1	1	
Birds							
	Red Bird of Paradise ..	2	0	0	0	0	
	Purple Headed Glossy Starling	2	0	0	0	0	
	Indian Pied Hornbill.. ..	0	3	3	2	1	See Figs. 5, 6 and 7.
	Grey Parrot	0	3	2	3	2	
	Grey Parrot	0	3	3	3	2	
	Amazon Parrot	0	2	1	1	1	
	Cockatoo	0	3	1	2	1	See Figs. 9 and 10.
	Citroon Crested Cockatoo ..	0	0	0	3	2	Fat in media.
	Cockatiel	0	3	2	2	1	See Fig. 8.
	Common Vulture	0	2	1	2	1	
	Occipital Vulture	0	3	1	1	0	Little medial change.
	Ruddy Sheld-Duck	0	2	2	1	1	
	Common Peafowl	0	2	2	1	1	
	Burmese Peafowl	0	3	2	1	1	
	Crested Curassows	0	0	0	3	2	See Figs. 3 and 4.
	Chilean Flamingo ..	0	2	3	1	1	First time observed in order See Fig 2.

1 = slight change D.I.T. = diffuse intimal thickening M.D. = medial degeneration
2 = moderate change F.I.T. = focal intimal thickening M.M.A. = medial mucoid accumulation
3 = marked change I.F.A. = intimal fatty accumulation

and two thirds of the thickness of the media. Grade 3 (Marked) : intimal thickening more than two thirds the thickness of the media.

Medial degeneration (Figs. 1, 2, 3, 4, 11). A process in which distortion and slitting of elastic fibrils is found, often with loss of medial thickness. Changes were graded as follows. Grade 1 : slight fraying of elastic fibrils only. Grade 2 : fraying and distortion of elastic fibrils, and their separation by mucoid or other material. Grade 3 : Grade 2 changes in more severe form.

DISCUSSION

In this study the accumulation of mucoid material in the media of the aorta and to a lesser extent the intima, has been a prominent feature. While small amounts of such substances form the normal ground-substance of arteries in both man and animals, the formation of large pools of mucoid material, which could be visualized in the media even by haematoxylin and eosin because of the accumulation of basophilic pools of mucoid material (Figs. 1 and 3), cannot be considered normal, especially as such changes were always found in association with degeneration of elastic tissue. Mucoid material in the intima was found only in small quantities in these animals, perhaps because it formed the ground material for collagen formation. There was no evidence that this substance had any special affinity for lipids. It is interesting to note that Taylor (1953) in a study of human aortae found the mucoid material they contained to be PAS-negative, but staining metachromatically with aqueous toluidine blue. In these animals PAS-positive streaks were found closely applied to degenerative elastic fibrils but, except in the case of the lion (Fig. 1), the mucoid material itself was PAS-negative, though staining metachromatically with toluidine blue, thus paralleling Taylor's findings. The significance of the PAS-positive mucoid material in the lion is not clear, but since the PAS reaction depends on available 1:2 glycol groupings (Taylor and Saunders, 1957) it may represent a partial breakdown of mucoid material. It seems unlikely that the aortic disease was related to the muscular dystrophy.

An important point is emphasized in such animals as the Crested curassows and the Citron-crested cockatoo, for in them marked medial degeneration and accumulation of mucoid material occurred without any change in the overlying intima (Figs. 3 and 4). Study of Table 2 will reveal that, when focal intimal thickening is present, medial degeneration is always associated, though this may be slight as in the occipital vulture. This is interesting in view of the observations of Lansing (1954) on human elastic tissue and its relation to intimal disease. He showed that medial disease precedes intimal disease, and may determine its site. In these animals medial degeneration and thinning were associated with focal intimal disease, whereas the media was not thinned when medial degeneration

occurred as an isolated lesion. Obviously no conclusions can be drawn about the sequence of events from the animals studied, but if medial disease precedes intimal disease in animals, it is surprising that severe medial disease can be found in autopsy material without any apparent changes in the overlying intima.

In assessing the evidence for the episodic nature of animal arteriosclerosis three processes seemed to be involved in these animals. The first concerns layering in the plaque. Elastosis, that is the splitting and reduplication of the internal elastic membrane with associated elastic tissue degeneration in the media, was a common finding (Fig. 11). Occasionally, however, an appearance akin to the "layering" described by Morgan (1956) was seen (Fig. 2). In this animal, a Chilean flamingo, the appearance of "layers" was heightened by the fact that the elastic tissue strands forming the layers were well preserved. It is however impossible to be sure that these appearances were not caused by elastosis, but it should be noted that also in this animal superficial and deep accumulations of lipid occurred separated by a lipid-free zone. The more common picture was, however, diffuse infiltration of lipid Fig. 7).

The second point concerns the presence of superficial foam-cell accumulations. Though marked in only one case, the presence of superficial foam-cell accumulations on the surface of a plaque and sharply demarcated from it (Fig. 8) would seem to be evidence in favour of an episodic rather than a continuous process.

In the third place mural thrombosis has to be considered. A small mural thrombus was found in one case, the Indian Pied Hornbill, but its appearance is sufficiently dramatic to warrant discussion (Figs. 5 and 6). Duguid (1948) believes that focal intimal thickening and intimal fatty accumulations, that is, atheroma, may occur in man secondary to the incorporation of small mural thrombi into the walls of arteries. He points out that the endothelium is not a fixed boundary but may grow over such thrombi. Whether this theory has any counterpart as an initiating mechanism in animal arteriosclerosis is impossible to say, but mural thrombi occurring on established plaques may represent another method of the episodic propagation of such a plaque. The appearances strongly favour this possibility.

Cartilage and pseudo-cartilage formation (Figs. 9 and 10) were not uncommon in the animals studied and have no counterpart in the human forms of arteriosclerosis (although ossification may occur in Mönckeberg's Sclerosis) even though man and long-lived birds such as parrots may have equivalent life spans. Also as pointed out by Fox (1933 a) ulcerated and thrombotic lesions of severe and lethal potentialities as they occur in man, are not found even in long-lived, overfed, under-exercised animals. Two interesting findings in the Korin Gazelle were the amount of elastic tissue degeneration in relation to the intimal plaque (Fig. 11) and the presence of large amounts of smooth muscle tissue in the proximal

part of the aorta (Fig. 12). This tissue occurred in blocks, and at the edges of the blocks elastic fibres were often found to be frayed. This may be a normal feature of game animals (order Artiodactyla) as it has been observed on several occasions in the aortae of animals shot in the wild state. Finally, it should be noted that in none of the animals was the arterial disease thought to have had an inflammatory or parasitic aetiology, although as Fox observed, both factors could occasionally produce arterial change.

CONCLUSIONS

An account is given of certain aspects of spontaneous aortic disease in 36 wild animals kept and dying in captivity (5 mammals and 31 birds). Mucoid changes similar to those described in human arterial disease were common and of importance in these animals. There was also some evidence that the arterial changes observed were the result of an episodic process similar to that described in certain cases of human arteriosclerosis. Certain morphological changes which supported this belief such as the presence of superficial foam-cell accumulations, mural thrombosis formation and "layering" in the plaque, are discussed. Other features peculiar to animal arteriosclerosis, such as the formation of cartilage, are noted.

ACKNOWLEDGMENTS

It is a pleasure to record my indebtedness to several people. Professor Alan Kekwick of the Middlesex Hospital conceived the idea of this investigation, and throughout it gave me much of his valuable time, advice and encouragement. The Prosectorial Committee of the Royal Zoological Society gave me permission to use material from the London Zoo, and Professor Osman Hill and Mr. Richard Fiennes, Pathologist to the Zoo, gave me full co-operation in obtaining it. Dr. Drew Thomson and Dr. Peter Andrews of the Bland-Sutton Institute of Pathology gave me much help with the histology.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Section of Lion's aorta showing diffuse intimal thickening and medial degeneration. The intima is at the top of the field, and is approximately seven cell layers thick. The internal elastic lamina is well marked (for PAS staining), and the underlying media is degenerate. There are collections of PAS-positive mucoid material (pink in the original section) in between ruptured and distorted elastic fibrils. P.A.S. × 100.

Fig. 2. Section of Chilean Flamingo's aorta showing focal intimal thickening and medial degeneration. The intima is on the left of the field, and is expanded into a layer thicker than the media. There are superficial and deep foam cell accumulations in the intima (which appear light) separated by a fibrous zone. There is some evidence of 'layering' at the base of the plaque. The underlying media is degenerate. Weigert-Van Gieson. × 50.

Figs. 3 and 4. Sections of Crested Curassow's aorta showing marked medial degeneration without changes in the overlying intima (at the top of the field). The media is degenerate throughout its thickness and this is evident even on H and E staining (Fig. 3), because of the accumulation of basophilic pools of mucoid material. Elastic tissue degeneration (Fig. 4) is marked. Fig. 3 H and E. × 85. Fig. 4 Weigert. × 95.

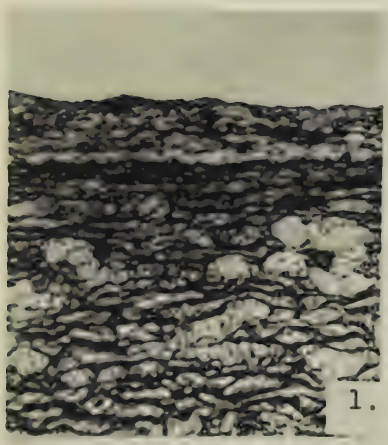
Figs. 5 and 6. Sections of Indian Pied Hornbill's aorta showing extensive intimal thickening. There is a mural thrombus on top of the diseased intima, and higher magnification (Fig. 6) shows this to be made up of blood-elements including nucleated erythrocytes (compare with erythrocyte in lumen at upper left part of field, Fig. 6). H and E: Fig. 5 × 85, Fig. 6 × 300.

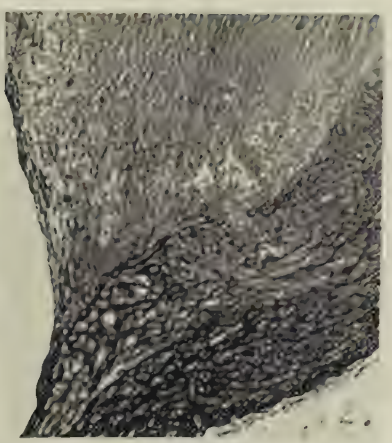
Fig. 7. Section of Indian Pied Hornbill's aorta (same animal as in Figs. 5 and 6) showing intimal accumulation of lipids. (Sudan IV × 28).

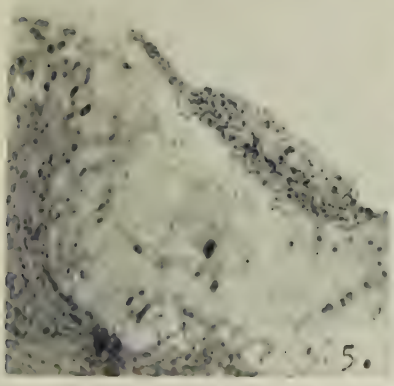
Fig. 8. Section of Cockatiel's aorta showing superficial foam cell accumulations on the surface of the intima (lumen at top of field, intima which is grossly thickened at bottom right). Note that the collection of foam cells is demarcated from the rest of intima. H and E. × 400.

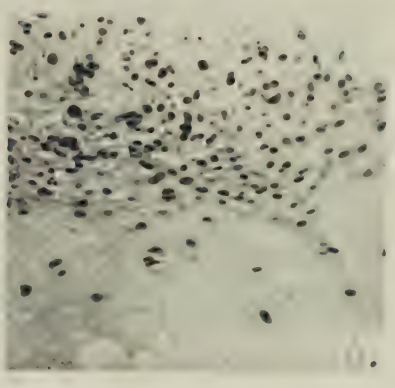
Figs. 9 and 10. Sections of Cockatoo's aorta showing cartilage and pseudocartilage formation in a thickened intima. The media begins towards the bottom of both fields. H and E: Fig. 9 × 95. Fig. 10 × 400.

Figs. 11 and 12. Sections of Korin Gazelle's aorta. Fig. 11 shows focal intimal thickening (at top left part of field), and elastic tissue degeneration. Fig. 12 shows the proximal part of the aorta in which blocks of smooth muscle with well marked nuclei on H and E staining, were found (here they appear much paler than the surrounding elastic tissue). Weigert-Van Gieson: Fig. 11 × 55. Fig. 12 × 40.









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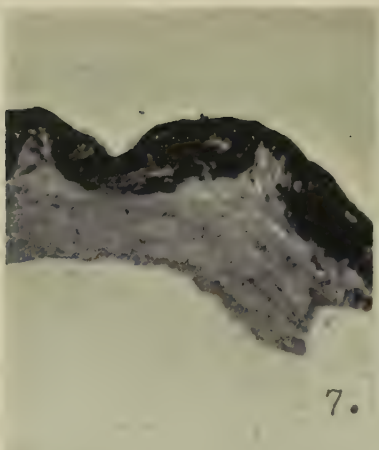

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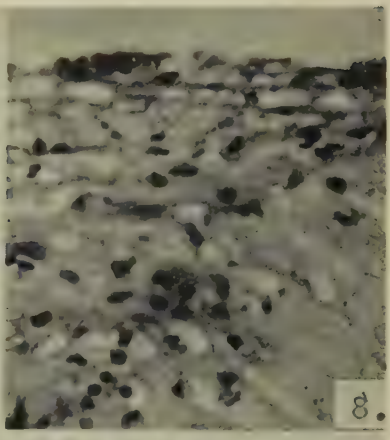

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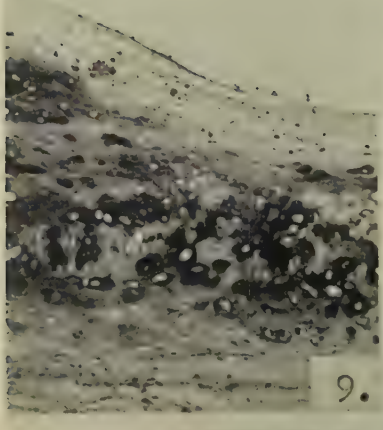

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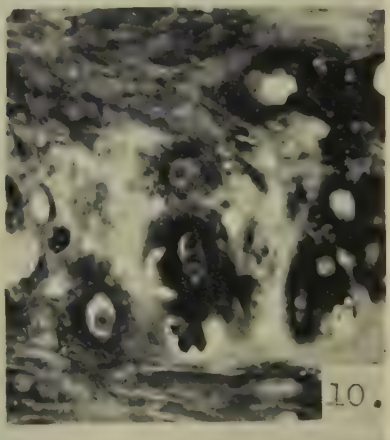

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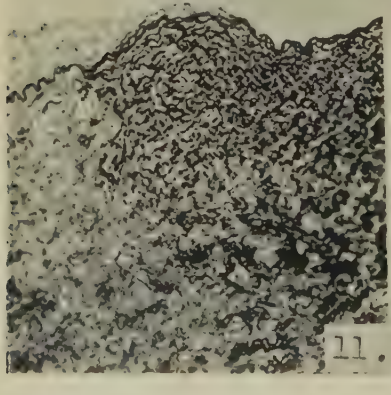

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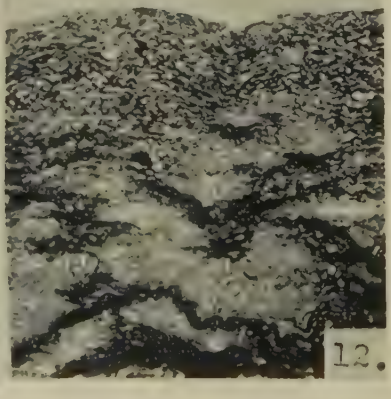

12.

CHEMOPROPHYLAXIS AGAINST BOVINE TRYPANOSOMIASIS

I. Duration of Protection from Prothidium, and Ethidium and R.D.2902 Suraminates, in an Area of High Tsetse Density

By

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INTRODUCTION

Watkins and Wolfe (1956) showed that some drugs structurally combining the phenanthridine with the pyrimidine nucleus were active against *Trypanosoma congolense* in mice. Of this type of compound Robson and Milne (1957) tested three in Zebu cattle, using heavy challenges of a syringe-passaged strain of *T. congolense*. Repeated inoculations of the organisms indicated that although the compounds varied slightly between themselves they protected the animals against infection for about four to six months. At about the same time Williamson and Desowitz (1956) gave details of the compounding of common bovine trypanocides with suramin (B.P.). These workers found that a single dose of Ethidium suraminate protected West African cattle for about 13 months. The animals were challenged frequently under laboratory conditions chiefly with *Glossina palpalis*, about 90 per cent of which were infected with *T. vivax*. Of other suraminate preparations tested some were less effective, but in many instances the prophylaxis from the suraminate notably exceeded the duration of protection from the corresponding agent not linked with suramin: moreover, when compounded with suramin comparatively much larger doses of an anti-trypanosomal drug could be administered without a correspondingly increased toxic effect.

This paper describes an experiment carried out under field conditions using some of the prophylactic drugs found most promising by the workers mentioned above.

MATERIALS AND METHODS

Cattle. Immature shorthorned East African Zebu heifers and bulls, and a few bullocks, which had not previously been in contact with tsetse, were used. Half the females were light coloured, varying from white to red, and half were dark brown or black. The males were similarly divided by colour.

Drugs. Ethidium bromide*, Prothidium*, and R.D.2902* were supplied gratis through the courtesy of Boots Pure Drug Company, Limited. Suraminates of Ethidium and R.D.2902 were prepared with suramin†, after the latter had been dried for 24 hours at about 130°C. by the method of Williamson (1957). Prothidium was prepared as a 2 per cent suspension in distilled water. The commercial product of standard formulation quinapyramine (prophylactic)‡ was used in the routine way. A sample

*Boots Pure Drug Co.
†Antrypol: I.C. (Pharmaceuticals) Ltd.
‡Antrycide Prosalt: I.C. (Pharmaceuticals) Ltd.

of the material, checked through the courtesy of the Imperial Chemical (Pharmaceuticals) Ltd., was found to conform to specification. The drugs were injected on the day they were prepared and details of the dosages used are given in Table 1. For the suraminates the dose recorded is that of the active component present. The weight of animals was rounded to the nearest five kg. and the volume of inoculum was given to the nearest 0·5 ml.

Trypanosome challenge. The experimental area, which lies near Lugala in the Busoga flybelt in southeast Uganda, is infested chiefly by *G. pallidipes*, though a few *G. brevipalpis* and *G. palpalis fuscipes* also occur. The intensity of the challenge, which was severe, has been described by Smith and Rennison (in preparation); the species of pathogenic trypanosomes present include *T. vivax, T. congolense* and *T. brucei* group.

Examinations. Thick, Giemsa-stained films of ear-blood from the cattle were made on alternative days between 7.00 and 8.30 a.m.; dewlap measurements were carried out weekly with a pair of calipers

Plan of experiment. Sixty-four cattle were allotted to four groups so that 16 animals were inoculated with each drug; further subdivisions were made to give eight animals for each level of drug, there being a dummy subgroup for quinapyramine (prophylactic). It was further arranged that each subgroup for drug level contained four light coloured and four dark animals, and these each comprised two males and two females. Random selection was used throughout in assigning the animals to the drug groups and subgroups. Details of the design are given in Table 1. Two untreated control groups, in each of which were a light coloured male and female and a dark male and female, were also exposed to infection.

Films were made from the animals beginning three weeks before the drugs were injected. The transverse thickness of the dewlaps of the cattle was measured before and immediately after they were injected with the prophylactic drugs. Four days later the animals, with one of the untreated control groups, were taken to the experimental site from the tsetse-free area in which they had been held. Three months after the work began the second batch of controls was introduced. When an animal was found to be infected it was brought within two days from the experimental area to a tsetse-free area and placed at random into one of three groups, treated respectively with Ethidium bromide (2·5 mg./kg.), Ethidium chloride (2·5 mg./kg.) and Berenil* (3·5 mg./kg.). No animal was treated with any of these drugs until trypanosomes had been found in at least two films of ear blood.

Treatment of data. Because the mean number of days of protection for each drug group tended to be approximately proportional to the standard deviation of the group, the logarithm of the number of days of protection was used when the main comparisons between the various drug preparations were made.

RESULTS

Before Exposure

No trypanosomes were detected in any animal in the period before the drugs were injected and before they were exposed to challenge.

*Farbwerke Hoechst Ag.

After Exposure

The control groups. The effect of challenge on the untreated cattle is shown in Table 1. Briefly, the average length of time from exposure to *Glossina* until trypanosomes were detected in films was about nine days for the animals which accompanied the main herd, and about 10 days for the controls introduced three months later. Three animals (65, 66, and 68), all of which showed mixed

TABLE 1

ZEBU CATTLE TREATED WITH VARIOUS PROPHYLACTIC DRUGS

Drug	*Dose mg/kg*	*Colour*	*Sex*	*Animal No.*	*Dewlap rupture weeks after injection*	*No. days after exposure tryps. first found in blood*	*Mean for group days*	*First species detected*
Ethidium suraminate	5	Light	M	1	9	73		*T. vivax*
			M	2	22	85		*T. vivax*
			F	3	14	99		*T. brucei*
			F	4	22	59		*T. vivax*
		Dark	M	5	9	109		*T. congolense* *T. vivax*
			M	6	9	79		*T. vivax*
			F	7	not recorded	139		*T. vivax*
			F	8	8	109	94·0	*T. vivax*
	10	Light	M	9	8	73		*T. vivax*
			M	10	8	79		*T. vivax*
			F	11	10	87		*T. vivax*
			F	12	9	95		*T. vivax*
		Dark	M	13	8	101		*T. vivax & congolense*
			M	14	14	>141		—
			F	15	6	85		*T. vivax*
			F	16	6	75	92·0	not recorded
Prothidium	2	Light	M	17	22	87		*T. vivax*
			M	18	22	79		*T. vivax*
			F	19	22	77		*T. vivax*
			F	20	22	47		*T. vivax*
		Dark	M	21	22	87		*T. vivax*
			M	22	22	67		not recorded
			F	23	22	51		*T. vivax*
			F	24	13	91	73·3	*T. vivax*
	4	Light	M	25	22	87		*T. vivax*
			M	26	22	113		*T. vivax*
			F	27	22	91		*T. vivax*
			F	28	22	73		*T. vivax*
		Dark	M	29	22	109		*T. vivax*
			M	30	22	69		*T. vivax*
			F	31	22	97		*T. vivax*
			F	32	22	99	92·2	*T. vivax*

Table 1 continued

Drug	*Dose mg/kg*	*Colour*	*Sex*	*Animal No.*	*Dewlap rupture weeks after injection*	*No. days after exposure tryps. first found in blood*	*Mean for group days*	*First species detected*
RD2902 suraminate	5	Light	M	33	22	103		*T. vivax*
			M	34	13	55		*T. vivax*
			F	35	22	91		*T. vivax*
			F	36	22	59		*T. vivax*
		Dark	M	37	9	125		*T. viva*
			M	38	22	129		*T. vivax*
			F	39	8	61		not recorded
			F	40	22	121	93·0	*T. vivax*
	10	Light	M	41	22	87		*T. vivax*
			M	42	5	135		*T. congolense*
			F	43	22	>141		—
			F	44	22	121		not recorded
		Dark	M	45	7	109		*T. vivax*
			M	46	5	79		*T. vivax*
			F	47	5	67		*T. vivax*
			F	48	7	93	104·0	*T. vivax*
Quinapyramine prophylactic	11·7	Light	M	49	22	49		*T. vivax*
			M	50	22	47		*T. congolense*
			F	51	22	53		*T. vivax*
			F	52	22	73		*T. vivax*
		Dark	M	53	22	53		*T. vivax*
			M	54	22	49		*T. vivax*
			F	55	22	67		*T. vivax*
			F	56	22	53	55·5	*T. vivax*
	11·7	Light	M	57	22	43		*T. vivax*
			M	58	22	47		*T. vivax*
			F	59	22	51		*T. vivax*
			F	60	22	>141		—
		Dark	M	61	22	75		*T. vivax*
			M	62	22	59		*T. vivax*
			F	63	22	53		*T. vivax*
			F	64	22	71	67·5	*T. vivax*
Untreated	First	Light	M	65	—	9		*T. vivax & brucei*
			F	66	—	9		*T. vivax & brucei*
		Dark	M	67	—	9		*T. vivax & brucei*
			F	68	—	9	9·0	*T. vivax*
	Second	Light	M	69	—	13		*T. vivax & brucei*
			F	70	—	11		*T. vivax*
		Dark	M	71	—	7		*T. vivax*
			F	72	—	9	10·0	*T. vivax*

T. brucei = *T. brucei* group. M = Male, F = Female. > 141 = 141 for analysis.

KEY TO TABLE 1

	Analysis (log transformation)		
	Df.	Mean sq.	F.
Drugs	3	0·1427	10·19***
Levels within drugs	4	0·0178	1·27
Colour	1	0·0162	1·16
Sex	1	0·0002	0·01
Error	54	0·0140	

infections with *T. vivax*, *T. congolense* and *T. brucei* group, died about 15 days after they were first found to be infected. *T. congolense* appeared only in blood films taken late in the course of infection, usually within one or two days of death. The other five animals (67, 69, 70, 71 and 72) were treated with anti-trypanosomal drugs, but cures were not obtained in three of them. Full details will be published in a subsequent paper. It appears from these data that virulent strains of the various species of trypanosomes exist in the experimental area. It is possible also that their pathogenicity is enhanced in some way by *G. pallidipes*, an effect noted previously by Lewis (1947).

Treated groups. The numbers of days which elapsed before organisms were found in the blood of the cattle treated with the various prophylactic drugs are recorded in Table 1. The period may be one day too long in each case because films were made on alternate days only. In most instances the organism first to appear was *T. vivax*.

The number of trypanosomes which could be found in films from control cattle very noticeably exceeded the numbers to be seen in those from animals which had received a prophylactic drug. It frequently occurred during the experiment that a positive film was obtained from an animal on a particular day but that trypanosomes could not be found in subsequent films. In one case, as many as 29 further examinations were negative after the organism had first been detected. Only about 50 per cent of the animals produced two consecutive positive films with an interval of one day between them. This effect was not apparently related to the various prophylactic drugs which the cattle received (Table 2). From these data it is clear that assuming the organisms are constantly present in the peripheral circulation, the probability of finding the pathogens known to be present in an animal, even after examining at least 200 fields of a thick blood film, is not high. It is obviously desirable to take blood films very frequently from experimental animals treated with any prophylactic drug and exposed to trypanosomes.

It will be observed from the analysis appended to Table 1 that there was a significant difference between the effectiveness of the drugs used. R.D.2902 suraminate afforded the longest protection, with a mean of about 99 days. Ethidium suraminate prevented the appearance of trypanosomes in the circulation for about 93 days, Prothidium for a mean of about 83 days and quinapyramine (prophylactic) for about 61 days. The difference between the test drugs

and the standard, quinapyramine (prophylactic), was unlikely to have been due to chance ($P<0.001$). Another experiment carried out simultaneously with the one described in this paper and using the same group of animals treated with standard formulation quinapyramine (prophylactic) as controls for a group treated with a modified formulation of this drug showed that the latter gave better protection, but both preparations were inferior in prophylactic power to those with the phenanthridinium base (unpublished data).

TABLE 2

FREQUENCY WITH WHICH NO TRYPANOSOMES WERE FOUND IN BLOOD FILMS ON ALTERNATE DAYS

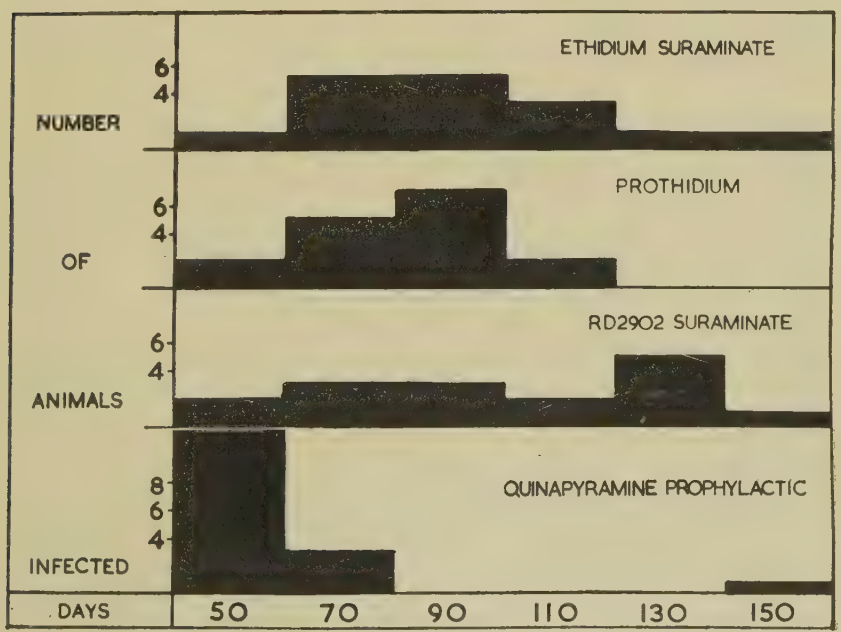
No. of consecutive slides in which no trypanosomes found after latter first detected	*Frequency*				*Total*
	Ethidium suraminate	*Prothidium*	*R.D.2902 suraminate*	*Quinapyramine (prophylactic)*	
0	10	10	6	9	35
1	1	0	2	3	6
2	2	0	1	0	3
3	2	1	1	1	5
4	0	1	0	0	1
5	0	1	1	0	2
6	0	1	0	2	3
>6	0	2	4	0	6
Total	15	16	15	15	61
Animals never positive	1 (No. 14)	—	1 (No. 43)	1 (No. 60)	3

It can also be seen from Table 1 that there was no significant difference in protection conferred by the two dosage rates of the various drugs. This rather surprising finding may have been due to the marked variation in prophylactic period between animals, irrespective of the prophylactic drug they had received; indeed, the larger dosage rate of the Ethidium suraminate protected slightly less well than the lower amount (Table 1). The results may, however, be an indication of a lack of independence between drugs and levels of the same drugs: they may arise from the transmission of drug-resistant tryanosomes between cattle. The distribution of the actual times of 'breakthrough' of the animals in the experiment is shown in Fig. 1. Examination of these data indicated that in the

case of the cattle treated with Prothidium and the two suraminates there was no departure from normality, but the numbers of animals involved were too few to permit a definite conclusion and it was deemed safer to make comparisons with log-transformed data. It would be unwise, in view of the component levels-within-drugs which is not significant, to stress any observed difference in the protection conferred by the dosage rates of the various drugs, but it seems reasonable, since the experiment was designed to permit comparisons, to accept that 4 mg./kg. Prothidium, and the greater dosage rates of the two suraminates, possibly gave significantly longer protection than 2 mg./kg. of Prothidium (Table 3). The lower dosage rate of Prothidium was unlikely to have conferred a longer protection than did quinapyramine (prophylactic). There was no significant variation between the two groups treated with quinapyramine (prophylactic) and, to increase precision, the results for the eight animals treated with the various levels of the test drugs (Table 3) were tested against the combined results for the 16 animals treated with quinapyramine (prophylactic).

Neither the sex nor the colour of an animal had any bearing on the prophylactic period (Table 1). None of the various interactions approached significance and all are pooled in the error term (Table 1).

Fig. 1



The distribution of times of 'breakthrough' with trypanosomes in small, short-horned East African Zebu cattle which had been injected with various prophylactic drugs and then exposed to a high density of *G. pallidipes*.

TABLE 3

COMPARISON OF PROTECTION CONFERRED BY VARIOUS PROPHYLACTIC DRUGS

Drug	*Dose mg/kg.*	*Average protection (approx.)*	*Chance of not being significantly better*					
			Ethidium suraminate		*Prothidium*		*R.D.2902*	
			5 mg.	*10 mg.*	*2 mg.*	*4 mg.*	*5 mg.*	*10 mg.*
Ethidium ..	5	94·0	–	0	0	0	0	0
suraminate ..	10	92·0	0	–	1/20	0	0	0
Prothidium ..	2	73·0	0	1/20	–	1/30	0	1/60
	4	92·0	0	0	1/30	–	0	0
R.D.2902 ..	5	93·0	0	0	0	0	–	0
suraminate ..	10	104·0	0	0	1/60	0	0	–
Quinapyramine .. (prophylactic)	11·7	62·0	1/200	1/200	0	1/200	1/200	1/200

0 = >1/20

Local and General Toxicity

The reaction produced in East African Zebu cattle by the newer drugs has been described by Robson and Cawdery (1958), and the observations reported in this paper agree with theirs. Irrespective of the dosage level, except in the case of the cattle treated with quinapyramine (prophylactic), a hard, plaque-like, oedematous swelling, frequently as large as 15·0 × 7·5 cm., developed in the dewlaps. Cultures of the preparations of Ethidium and R.D.2902 suraminates and of Prothidium prepared for injection revealed no bacteria likely to have produced this effect and it may be assumed that the reaction was due to the drugs themselves. The lesion gradually regressed until a circumscribed, protuberant and somewhat pendulous swelling remained often showing a patch of necrotic skin which eventually eroded completely. Pus-like material, containing many dead neutrophils and small round cells in a fluid which appeared macroscopically to be stained the colour of the drug, was discharged. Although frequent manipulation may have speeded the rupture it is unlikely to have caused it. The dewlap ulceration which followed rupture was unsightly, painful and healed slowly. Finally there remained a piece of puckered skin about five cm. long with, in instances, adhesions to the underlying tissues. The sites in the dewlaps of the animals injected with Ethidium suraminate burst sooner than those inoculated with R.D.2902 suraminate, with which drug also fewer complete ruptures were recorded, the proportions being 13 out of 15 and eight out of 16 respectively (Table 1). Rupture occurred in only one animal treated with Prothidium and in none of the cattle dosed with

quinapyramine (prophylactic). It is apparent from Table 4 that the swelling at the inoculation site produced by the injection of Prothidium was greater than was the case with the suraminates, but the lesions also decreased in size more quickly and after about four weeks they were scarcely more prominent than those in cattle inoculated with quinapyramine (prophylactic). The terminal effect differed in that at the site of injection in the animals treated with Prothidium the ventral edge of the dewlap was distorted and fissured, the lesion being about two or three inches long, whereas the quinapyramine (prophylactic) injections produced the familiar nodule only.

TABLE 4

SWELLING PRODUCED IN THE DEWLAPS

Drug	*Dose mg/kg.*	*No. of animals*	*Injection to rupture: mean no days*	*Mean transverse thickness of dewlap swelling: mm.*						
				pre-injection	*post-injection*	*after one week*	*after four weeks*	*after eight weeks*	*after twelve weeks*	*after sixteen weeks*
Ethidium suraminate	5	8	101·6	7·2	14·6	37·5	46·4	51·0	38·5	31·5
	10	8	61·6	7·1	14·1	32·4	49·9	58·8	45·5	30·9
Prothidium	2	8	147·2	8·6	15·0	51·1	37·0	28·5	18·4	21·5
	4	8	155	7·9	15·0	66·9	36·0	28·5	18·3	21·7
R.D.2902 suraminate	5	8	123·9	7·1	14·0	32·6	40·0	40·2	32·1	33·5
	10	8	84·8	7·8	19·1	40·5	53·8	38·0	30·0	32·1
Quinapyramine (prophylactic)	11·7	16	155	6·7	13·0	30·5	28·8	22·1	22·6	23·0

A possible explanation of the failure of the suraminates injected at a rate of 10 mg./kg. to protect longer than at a rate of 5 mg./kg. may be that bursting at the site of inoculation occurred comparatively quickly with the higher doses. Covariance analysis of the data (Table 1) suggested, however, that the prophylactic period from Ethidium and R.D.2902 suraminates was not significantly influenced by early rupture at the site of injection.

Some generalized toxicity in the animals injected with the suraminate preparations may have been indicated by an unthriftiness which passed off slowly; the unthriftiness was not exhibited by the cattle treated with quinapyramine (prophylactic).

Drug Resistance

As already stated, some of the control animals were not cured by drug therapy and a similar result was experienced with animals in the groups which had received the various prophylactic drugs. Full details of these findings will be presented in a subsequent paper.

DISCUSSION

The cattle in this experiment were under very heavy challenge and all the drugs investigated would probably protect animals

for much longer periods in a lighter challenge. Nevertheless, the results of this investigation seem at variance with those recorded by both Robson and Milne (1957) and Whiteside and Lyttle (1957); these workers reported the absence of trypanosomes from the peripheral circulation and lymphatic nodes of Zebu cattle for about four months after injection of a single dose of 2 mg./kg. of Prothidium. Recent work, by Robson and Cawdery (1958), and by Tennant (1958) which indicates an average protection of about 70 days from 2 mg./kg. Prothidium in an area of "medium" challenge, agrees substantially with what is reported here.

The results indicate that the suraminate preparations could not be employed in the field by inoculation into the dewlap although they gave somewhat longer protection than Prothidium (4 mg./kg.), which itself was superior in prophylaxis to the standard formulation quinapyramine (prophylactic) on the average by about three weeks. The suraminates would probably be the drugs of choice if the serious reactions they produced could be diminshed by the use of other routes or sites of injection and if they were not too expensive. Some unpublished work, however, indicates that in other subcutaneous sites the response is equally severe, and the intramuscular inoculation of Ethidium suraminate also provokes a most serious reaction in East African Zebu stock. It is evident, therefore, that permanent muscle damage with consequent meat loss through condemnation is likely to occur. Possibly the severity of the reaction might be decreased by injecting multiple small doses.

The large variation noted by others (Davey, 1950; Fiennes, 1953) in the duration of protection, which can happen in cattle treated with quinapyramine (prophylactic), also occurred with all the drugs used in this experiment. To prevent the possible development of drug resistant strains both in cattle and in the tsetse population, wide limits must, therefore, be set for the assumed period of complete protection. The distribution of the times of 'breakthrough' of the animals in this experiment cannot be clearly discerned because of the small numbers employed. As indicated, although it was easily proven that the test preparations were superior to the standard, considerable difficulty arose when it was necessary to decide whether a given dose of one drug was more or less efficacious than another. It would appear that in future work when testing a prophylactic drug against bovine trypanosomiasis, each group should consist of about 30 animals in an endeavour to determine the type of distribution of 'breakthrough' times to be expected with any drug likely to be widely employed. If the numbers are so increased the prediction would rest on a basis more secure than is the case at the present moment. The mean of the 'breakthrough' times for the first few animals in a group treated with a prophylactic drug may be atypical for many reasons. The use of this mean to determine when the second dose of drug should be given seems to be unsatisfactory in that it sacrifices most of the information which

can be obtained on the form of the 'breakthrough' curve. Since the distribution must depend, to some extent at least, on the absorption rates of relatively insoluble drugs from a tissue depot, and their excretion, the form of the curve may well vary for different agents.

CONCLUSIONS

Groups of small, shorthorned East African Zebu cattle injected with Prothidium, the suraminates of Ethidium and R.D.2902 and standard formulation quinapyramine (prophylactic), were exposed to a high density of *G. pallidipes*.

Five and 10 mg./kg. of the suraminate preparations and 4 mg./kg. of Prothidium were superior in protective power to quinapyramine (prophylactic). The latter was possibly not less potent prophylactically than 2 mg./kg. of Prothidium.

All the preparations tested induced serious swellings at the site of injection in the dewlap but the reaction to Prothidium was the least severe.

The sex and colour of an animal appeared to play no part in determing the duration of protection.

ACKNOWLEDGMENTS

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THE PRODUCTION IN MICE OF ACTIVE IMMUNITY AGAINST *PASTEURELLA HAEMOLYTICA*

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INTRODUCTION

In an earlier publication (Smith, 1958) a means of producing true infections of *Past. haemolytica* in mice was described in which resistance to the experimental infection was depressed by granular mucin injected intraperitoneally together with the organisms. It was demonstrated that mice could be protected passively by immune antisera against such a method of challenge.

This work has now been extended to include a study of the protective effects of vaccination in mice, and the present paper records certain observations on this aspect of immunity.

MATERIALS AND METHODS

In the earlier publication referred to above detailed descriptions were given of the technical procedures and of the materials used. They cover the preparation and adjustment of bacterial suspensions for inoculation, the method of performing viable counts and the method of intraperitoneal challenge using organisms and mucin.

The same strain of *Past. haemolytica* (No. 7157H) originally isolated in 1954 from a lamb which died of a *Past. haemolytica* septicaemia as described by Stamp, Watt and Thomlinson (1955), was used for all vaccination and challenge purposes in the present series of experiments. A culture made from the heart blood of an experimentally infected mouse was freeze-dried in ampoules which were stored and used as required.

Preparation of Vaccines.

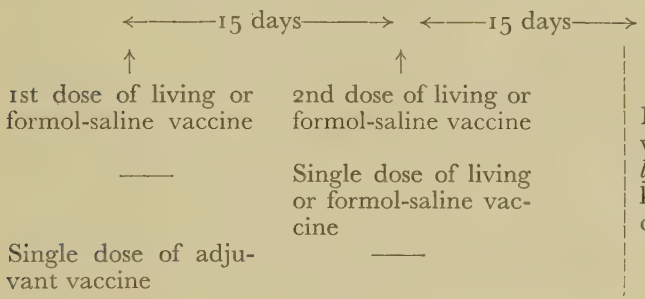
Living vaccine. This consisted of a 24 hour infusion broth culture injected in a dose volume of 0·2 ml. of a 1/5 broth dilution. Such a dose was known to be sub-lethal for mice although it contained 40 to 50 million viable bacteria.

Formol-saline vaccine. Seventeen-hour cultures on 5 per cent. sheep blood agar were suspended in normal saline and sterilised by washing twice and resuspending to the opacity of Brown's Tube 6 in 0·5 per cent. formol-saline. Suitable tests for purity and sterility were included. A single batch of this vaccine was prepared and stored at +4°C. for use in all the following experiments. The dose volume per mouse was 0·2 ml.

Adjuvant vaccine. The method of preparation consisted of a modification of the procedures used by Live (1949). A portion of the formol-saline vaccine described above was treated as follows. To each 1·0 ml. volume was added 0·027 ml. of sterile 10 per cent. sodium bicarbonate solution and 0·25 ml. of sterile 4 per cent. potassium aluminium sulphate solution. The mixture was allowed to stand overnight at +4°C., after

which the flocculated bacteria were washed and resuspended in 0·5 per cent. formol-saline. One volume of this suspension was then added to 4 volumes of sterile oil-Falba mixture which itself consisted of Bayol F* and Falba** in the proportion of 4 volumes to 1 volume. This final mixture was thoroughly emulsified. The dose per mouse was 0·05 ml. and the concentration of the vaccine was such that this volume contained the same number of dead bacteria as did a single dose of the formol-saline vaccine.

Vaccination and challenge schedules. Swiss white mice were used and at the beginning of each experiment they were 4 to 5 weeks old. The sexes were evenly distributed where necessary. The time relationships of the vaccination and challenge procedures employed are illustrated below.


←——15 days——→ ←——15 days——→
1st dose of living or formol-saline vaccine
2nd dose of living or formol-saline vaccine
Mice challenged with *Past. haemolytica* in mucin, or killed for serum collection.
——
Single dose of living or formol-saline vaccine
Single dose of adjuvant vaccine
——

Collection and use of mouse sera. Fifteen days after the completion of vaccination sera were taken from mice for the purpose of demonstrating either agglutinin or protective antibody. The sera were separated from bloods collected according to the method of Evans and Perkins (1954). They were stored at +4°C. and were used within 3 days. None was inactivated.

Each agglutination test was performed on a pool of equal volumes of 6 individual mouse sera. No assessment was made of the degree of accuracy to which this procedure indicated true average agglutinin titres The agglutination tests were carried out using 0·4 ml. volumes of two-fold serum dilutions in the presence of equal volumes of a stock formolised suspension of strain No. 7157H diluted to the opacity of Brown's Tube 3·5. Titres were read after 2 hours incubation at 55°C.

In the case of sera to be examined for protective antibody content no attempt was made to pool precisely equal volumes from individual mice. The control serum was separated from the bulked bloods of 56 two-month old untreated mice and was sterilised by filtration. The two immune sera were obtained and prepared in a similar manner from 2 vaccinated groups of 36 mice each.

Passive immunity tests and non-specific protection. In the serum protection experiment dilutions of immune and control mouse sera were injected intraperitoneally in dose volumes of 0·5 ml. per mouse, 5 to 6 hours before challenge with *Past. haemolytica* in mucin.

*Bayol F is a light mineral oil supplied by the Esso Petroleum Co. Ltd.

**Falba is a lanolin-like substance supplied by Pfaltz and Bauer, Inc., New York, U.S.A.

In order to determine whether the methods used might produce a non-specific immunity in mice the following procedures were carried out 5 to 6 hours before challenge, (*a*) the intraperitoneal insertion and withdrawal of a hypodermic needle; (*b*) the intraperitoneal injection in sterile 0·5 ml. amounts of normal saline*, pH 6·75, veronal-saline buffer, 1/10 dilution of normal mouse serum in normal saline, respectively. All fluids introduced into the peritoneal cavity were first heated to approximately 37°C.

RESULTS

Production of Active Immunity

Five groups of 58 mice each were vaccinated by the methods shown in Table 1 and a 6th group of the same size was left untreated for control purposes. All vaccines were administered intraperitoneally with the exception of the adjuvant vaccine which was injected subcutaneously on the inner aspect of the thigh.

Daily weighings of mice showed that injection of the formol-saline vaccine caused a loss of weight in 24 hours averaging 1·7 g. per mouse. In the same period the injection of living vaccine resulted in an average loss of 0·8 g. per mouse while adjuvant vaccine merely produced a temporary suspension of weight increase. These disturbances to the mice were apparently rapidly overcome, and at the time of challenge each of the 6 groups of 58 showed an average mouse weight within the range of 25·6 g. and 26·2 g.

TABLE 1

PRODUCTION OF ACTIVE IMMUNITY USING VARIOUS VACCINATION PROCEDURES

			Deaths in groups of 26 mice using 2 challenge levels		*Total deaths*	*Agglutinin titres*
			39 × 10⁶ organisms	*19·5 × 10⁶ organisms*		
Route and method of vaccination	I.P.	1 dose living vaccine	9	6	15/52	1/16
		2 doses living vaccine	1	0	1/52	1/1024
		1 dose formol-saline vaccine	3	2	5/52	1/64
		2 doses formol-saline vaccine	1	0	1/52	1/512
	S.C.	1 dose adjuvant vaccine	17	13	30/52	1/256
Unvaccinated controls			25	23	48/52	0

I.P.—intraperitoneal
S.C.=subcutaneous
Challenge doses injected intraperitoneally with mucin

*This was prepared as a 0·85 per cent. solution of sodium chloride in tap water.

Six mice in each group were killed in order to collect serum for agglutination tests. The remaining 52 in each group were divided into 2 sub-groups of 26 mice each since 2 challenge doses were used. The results recorded in Table 1 show that each method of vaccination protected mice against challenge with *Past. haemolytica* in mucin and in some cases the protection was very strong. Comparison of the figures for agglutinin titres and deaths in mice receiving single doses of living or formol-saline vaccine with those for mice receiving adjuvant vaccine suggests a lack of correlation between agglutinin production and degree of protection.

Demonstration of Humoral Protective Antibody

Three groups of mice were used in the way already described to obtain sera for use in passive mouse protection tests. The first group had previously received one dose of formol-saline vaccine, the second two doses and the third group had not been vaccinated. Serum pools from each of the 3 groups were injected intraperitoneally into mice in saline dilutions of 1/2 and 1/20, and in dose volumes of 0·5 ml.

Subsequent challenge with a single dose level of organisms demonstrated conclusively that sera from both groups of vaccinated mice protected at the 1/2 dilution, and also that the serum from mice which had received 2 doses of vaccine protected at the 1/20 dilution. The results of this experiment are shown in Table 2.

TABLE 2

DEMONSTRATION OF PROTECTIVE ANTIBODY IN SERA TAKEN FROM VACCINATED MICE

		Deaths produced by challenge in mice pretreated I.P. with doses of mouse sera	
		0·5 ml. 1/2 serum	*0·5 ml. 1/20 serum*
Sera obtained from mice previously treated as shown	1 dose formol-saline vaccine I.P.	0/12	3/12
	2 doses formol-saline vaccine I.P.	0/12	0/12
	Unvaccinated	10/20	11/20

I.P. = intraperitoneally

All mice challenged intraperitoneally with 21 × 10^8 viable bacteria in mucin 5 to 6 hours after injection of serum

Comparison of Intraperitoneal and Subcutaneous Routes of Vaccination

A single dose of formol-saline vaccine was injected intraperitoneally into mice of one group and subcutaneously in the dorsal

region of mice of a second group. A third group did not receive vaccine.

Table 3 shows the results obtained by challenging these mice with 2 dose levels of *Past. haemolytica*. The test was read at the 18th hour following challenge, and again at 72 hours after which time no further deaths occurred. It is clear that while intraperitoneal vaccination gave strong protection from death, subcutaneous vaccination afforded little or none although it undoubtedly did increase the survival time. In addition, a pool of serum from six mice vaccinated intraperitoneally showed a higher agglutinin titre than did a similar pool taken from subcutaneously vaccinated mice.

Comparison of Intraperitoneal and Intravenous Routes of Vaccination

The general design of this experiment was similar to that of the previous one. The results are set out in Table 4 and show that with regard to production of immunity and of agglutinin no significant difference between the two methods of vaccination could be detected.

Although no exact comparison with previous experiments is possible it is noticeable that the levels of protection and agglutinin produced by intraperitoneal vaccination in this experiment were somewhat lower than might have been expected. The type of agglutination was also unusual in that it was of a rather incomplete nature. This was the last experiment in which formol-saline vaccine was used and by this time the vaccine was almost four months old.

TABLE 3

COMPARISON OF INTRAPERITONEAL AND SUBCUTANEOUS ROUTES OF VACCINATION

		Vaccinated I.P.		*Vaccinated S.C.*		*Unvaccinated controls*	
Hours after challenge		*18*	*72*	*18*	*72*	*18*	*72*
Deaths in groups of 19 mice challenged as shown	40×10^6 organisms	1	7	7	17	15	19
	20×10^6 organisms	0	2	8	17	14	19
Agglutinin titres		1/64		1/8		0	

I.P. = intraperitoneally
S.C. = subcutaneously
Challenge doses injected intraperitoneally with mucin

Immunity not related to Antibody

In carrying out studies on immunity a strong suggestion was obtained that injection of certain fluids devoid of antibody into the peritoneal cavities of mice was capable of increasing resistance to intraperitoneal challenge with *Past. haemolytica* 5 to 6 hours later.

In regard to the passive immunity test recorded in Table 2 it will be seen that approximately 50 per cent. mortality was obtained in control mice which had received normal mouse serum several hours before challenge with 21 x 10^6 organisms. However the deaths among controls in those experiments recorded in Tables 1, 3 and 4 indicate that smaller doses of organisms may be capable of producing considerably higher mortality in mice not pretreated with normal serum.

TABLE 4

COMPARISON OF INTRAPERITONEAL AND INTRAVENOUS ROUTES OF VACCINATION

	Deaths in groups of 22 mice using 2 challenge levels		*Agglutinin titres*
	34 × 10^6	*17 × 10^6*	
Vaccinated I.P.	15	6	1/32
Vaccinated I.V.	18	7	1/32
Unvaccinated controls	20	20	0

I.P.=intraperitoneally
I.V.=intravenously
Challenge doses injected intraperitoneally with mucin

TABLE 5

PRODUCTION OF NON-SPECIFIC RESISTANCE BY VARIOUS TREATMENTS

		Deaths in groups of 10 mice challenged at 4 levels				*Total deaths produced by 2 smaller challenge doses*
		27 × 10^6 organisms	*13·5 × 10^6 organisms*	*6·75 × 10^6 organisms*	*3·3 × 10^6 organisms*	
Mice pretreated as shown, 5 to 6 hours before challenge	0·5 ml. 1/10 mouse serum I.P.	10	7	4	2	6/20
	0·5 ml. normal saline I.P.	9	6	0	0	0/20
	0·5 ml. veronal-NaCl buffer I.P.	9	9	5	2	7/20
	Needle trauma alone	10	10	8	3	11/20
Untreated controls		9	10	9	8	17/20

I.P=intraperitoneally
Challenge doses injected intraperitoneally with mucin

To investigate the matter further an experiment was performed in which 3 groups of mice received intraperitoneal injections of 1/10 mouse serum, normal saline or veronal-saline buffer respectively 5 to 6 hours before intraperitoneal challenge with *Past. haemolytica* in mucin. At the same time mice of a fourth group were stabbed intraperitoneally with a hypodermic needle. A fifth group consisted of untreated control mice. These 5 groups were divided into subgroups to allow for challenge with 4 dose levels of organisms. The results are shown in Table 5.

Injection of each of the three fluids resulted in increased resistance to challenge, but these protective effects were apparent only in mice which received the lower doses of organisms and were obliterated by the higher challenge levels. The trauma produced by a needle may also have resulted in some increased resistance but a further experiment would have been required to decide this matter conclusively.

DISCUSSION

One of the purposes of this paper has been to show that immunity to *Past. haemolytica* can be produced in mice by several different types of vaccine. Active immunity was readily demonstrated by intraperitoneal challenge with organisms suspended in mucin and all of the vaccination procedures investigated were protective. The methods described should facilitate the study of immunity to *Past. haemolytica,* particularly with regard to the immunogenic relationships between different strains. In the present series of experiments no attempt has been made to compare the immunising potencies of the various vaccines under test. Protection has been assessed at certain times after vaccination without regard to measurement of the rise, fall and duration of immunity, or to the effect of dose of vaccine. In the case of comparative studies on routes of vaccination conclusions have been drawn in strict relation to the particular experimental conditions used.

The ease with which protective antibody could be demonstrated in the sera of vaccinated mice by passive immunisation tests encourages the hope that sera from vaccinated sheep will also be capable of protecting mice. It must be admitted however that the doses of vaccine given to mice were comparatively large.

While it is certain that humoral antibody was concerned in the immunity produced by vaccination, the possibility that non-specific protective mechanisms may also have played a part at the time of challenge has not been eliminated. It is conceivable that substances giving rise to non-specific protection may be present in dead *Past. haemolytica,* and such a possibility cannot be adequately controlled in active protection tests. However, in view of the fairly long period between vaccination and challenge the effects of such bacterial substances were probably of minor importance if indeed they existed at all. The same applies to any local peritoneal immunity which

may have resulted initially from injecting the vaccine by the intraperitoneal route, and this is borne out by the fact that no significant difference could be observed between the efficiencies of intraperitoneal and intravenous vaccination against intraperitoneal challenge.

The degrees of protection afforded by intraperitoneal and subcutaneous vaccination differed strikingly, and this gives rise to speculation on the possible significance of route of vaccination against *Past. haemolytica* in the sheep.

Pfeiffer and Issaeff (1894) showed that certain substances such as broth and peptone solutions when injected intraperitoneally into guinea-pigs resulted in increased resistance to intraperitoneal challenge with cholera vibrios. Working with streptococcal infections in rabbits, Gay and Morrison (1923) found that the intrapleural injection of certain fluids, including broth, increased resistance to intrapleural challenge 24 hours later. This phenomenon was associated with an accumulation of macrophages in the pleural cavity. In the present study the protection against experimental *Past. haemolytica* infection produced by pretreatment of mice with fluids, including normal saline and dilutions of normal mouse serum was considerable. This protection was easily obliterated by increasing the challenge doses however and these increased challenge doses were known from previous work to have been readily capable of demonstrating true passive antibody immunity. These observations have considerable practical importance in studying immunity against *Past. haemolytica* infections in mice. In passive protection tests in which serum dilutions are injected into the peritoneal cavity a few hours before intraperitoneal challenge one must use challenge doses large enough to overcome the non-specific resistance produced in control mice. In active immunity experiments smaller challenge doses are satisfactory.

CONCLUSIONS

Various methods of vaccination have been found to produce demonstrable active immunity in mice against intraperitoneal challenge with *Past. haemolytica* in mucin.

The demonstration of immune antibody in the sera of vaccinated mice has been achieved by means of passive mouse protection tests.

Under the experimental conditions used, intraperitoneal vaccination produced a higher level of protection than did subcutaneous vaccination. In this respect however no difference could be shown between the efficiencies of intraperitoneal and intravenous vaccination.

The injection of certain fluids including normal saline into the peritoneal cavities of mice five to six hours before intraperitoneal challenge with *Past. haemolytica* in mucin has been shown to be capable of protecting from death. This effect is easily obliterated by increasing the challenge doses.

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I.C.I. Research leads to enhanced efficiency of Phenothiazine

That particle size can influence the anthelmintic efficiency of phenothiazine was first shown in 1950 by workers in Australia as a result of investigations carried out in collaboration with I.C.I. veterinarians, using a fine grade of drug specially produced in the I.C.I. laboratories.

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